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Award Number: W81XWH-10-1-0047

TITLE: Dietary Regulation of PTEN Signaling and Mammary Tumor Initiating Cells: Implications for Breast Cancer Prevention

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REPORT DATE: July 2012

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD/MM/YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From – To)*
July 2012	Annual Summary	01 January 2010-31 July 2012

4. TITLE AND SUBTITLE:

Dietary Regulation of PTEN Signaling and Mammary Tumor Initiating Population: Implications for Breast Cancer Prevention

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0047

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Omar M Rahal and Rosalia CM Simmen

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

AR Children's Hospital
Research Institute
Little Rock, AR 72202

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES):
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

In the present grant, I demonstrated a linear pathway by which soy isoflavone genistein (GEN)-mediated increase in PTEN nuclear localization initiates an autoregulatory loop involving PTEN-dependent increases in p53 nuclear localization and PTEN/p53 functional interactions to enhance PTEN expression resulting in mammary epithelial cell differentiation. I also investigated the effect of lifetime intake of soy protein (SPI) diet relative to casein (CAS) diet on mammary stem cell (MaSC) population and tumor formation in MMTV-Wnt1-Transgenic (Wnt1-Tg) female mice. We report that mammary tumor incidence was lower in the SPI-fed group by age 8 months. Mammary epithelial cells from SPI-fed Wnt1-Tg mice exhibited fewer MaSCs; decreased ability to form mammospheres in culture, lower mammary outgrowth potential when transplanted into cleared fat pads, and reduced accumulation of cancer stem cells. Gene array of the MaSC-enriched population demonstrated a stem cell-like expression pattern and markedly suppressed expression of inflammatory and metastasis-associated genes with dietary SPI exposure. SPI effects on MaSCs and tumor formation in Wnt1-Tg mice were recapitulated by dietary GEN. Our findings suggest an association between dietary regulation of mammary stem/progenitor cells and inhibition of tumor susceptibility and highlight diet-regulated, stem cell-associated genes for potential application in breast cancer therapy.

15. SUBJECT TERMS
Mammary stem cells, diet, breast cancer prevention, PTEN, p53, Wnt-transgenic

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT:** UU	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT: U	**b. ABSTRACT:** U	**c. THIS PAGE:** U		42	USAMRMC
					19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION

Epidemiological studies demonstrate an inverse association between high consumption of soy foods and breast cancer risk among Asian women compared to Western counterpart (1). This association was not related to classical risks for breast cancer such as mammographic density and estrogen levels, suggesting other mechanisms are involved, yet to be discovered. In this regard, our team has shown that dietary intake of soy protein isolate (SPI) or genistein (GEN)-supplemented casein diet protects against chemical-induced mammary tumorigenesis in rats fed these diets relative to those fed the control casein (CAS) diet (2-4). Mechanistically, protection by dietary SPI and GEN was mediated, in part, through upregulation of tumor suppressor PTEN (3, 5), and inhibition of oncogenic Wnt signaling pathway (4). The Wnt signaling pathway is necessary for normal mammary gland development (6); however its overexpression in the mammary gland such as in mouse mammary tumor virus Wnt1-transgenic (MMTV Wnt1-Tg) mice, results in spontaneous mammary tumor formation (7). Expansion of mammary progenitors (8-9) and mammary stem cell (MaSC)-enriched population ($CD29^{hi}CD24^{+}$) (10) in mammary glands of Wnt1-Tg mice, indicates an essential role for mammary stem/progenitor cells in Wnt1-induced mammary tumorigenesis. Indeed, mammary tumors from Wnt1-Tg mice contain a small population of cancer stem cells (CSCs) (Thy1+CD24+) with high tumorigenic activity upon transplantation (11). Further, ectopic overexpression of Wnt1 confers radiation resistance (12) and tumor initiating potential (13) to mammary progenitors. A linkage between PTEN and Wnt signaling pathways in mammary tumorigenesis is suggested by the findings that PTEN deficiency results in decreased latency of tumor formation in Wnt-1 transgenic mice (14) and when overexpressed, PTEN protects against Wnt1-induced mammary tumorigenesis (15). Therefore, ***we hypothesize that dietary soy protein isolate protects against Wnt-induced mammary tumorigenesis by up-regulating PTEN signaling and decreasing normal mammary stem cell population, thus preventing the conversion of normal stem cells to cancer stem (tumor initiating) cells.***

BODY

The main goal of the present grant was to understand the implications of dietary regulation of tumor suppressor PTEN signaling and mammary stem cells on breast cancer. We used the well-characterized Wnt1-Tg mouse model of breast cancer (7) where spontaneous mammary tumor formation is preceded by dysregulation of mammary stem (10) and progenitor cells (8-9, 12) leading to conversion into CSCs (11, 13). The current grant is composed of two Specific Aims. Aim 1 identified a novel mechanism of dietary prevention where *soy isoflavone* GEN mediates a crossregulation between the tumor suppressors PTEN and p53 in human mammary epithelial cells (MECs) to promote cell differentiation and cell cycle arrest by increasing the nuclear PTEN pool. Mammary stem cells (MaSC) are required for tissue homeostasis and epithelial expansion during development; however, dysregulation of MaSC self-renewal such as by Wnt1 overexpression leads to neoplastic transformation. To date, *in vivo* dietary regulation of MaSC and its direct association with breast cancer has not been evaluated. Therefore, Aim 2 evaluated the effect of lifetime intake of soy protein (SPI) or genistein (GEN) diet relative to the control casein (CAS) diet on mammary stem cell (MaSC) population and its correlation to tumor formation in MMTV-Wnt1-Transgenic (Wnt1-Tg) female mice.

Tumors suppressors PTEN and p53 are commonly lost or mutated in several cancers including breast cancer (16). In the first aim, I evaluated the hypothesis that *soy isoflavone*

genistein (GEN) mediates the crosstalk between the tumor suppressors PTEN and p53 in human mammary epithelial cells (MECs) to promote cell differentiation and cell cycle arrest by increasing the nuclear PTEN pool. This published work (5) describes a linear pathway by which GEN-mediated increase in PTEN nuclear localization initiates an autoregulatory loop involving PTEN-dependent increases in p53 nuclear localization and PTEN/p53 functional interactions to enhance PTEN expression resulting in cell differentiation (Figure1). The implications of my findings go beyond nutrition and breast cancer as I provided the first direct evidence that PTEN, mainly known to be a phosphatase, can regulate its own expression in MECs by using siRNA, ChIP and promoter assays (5).

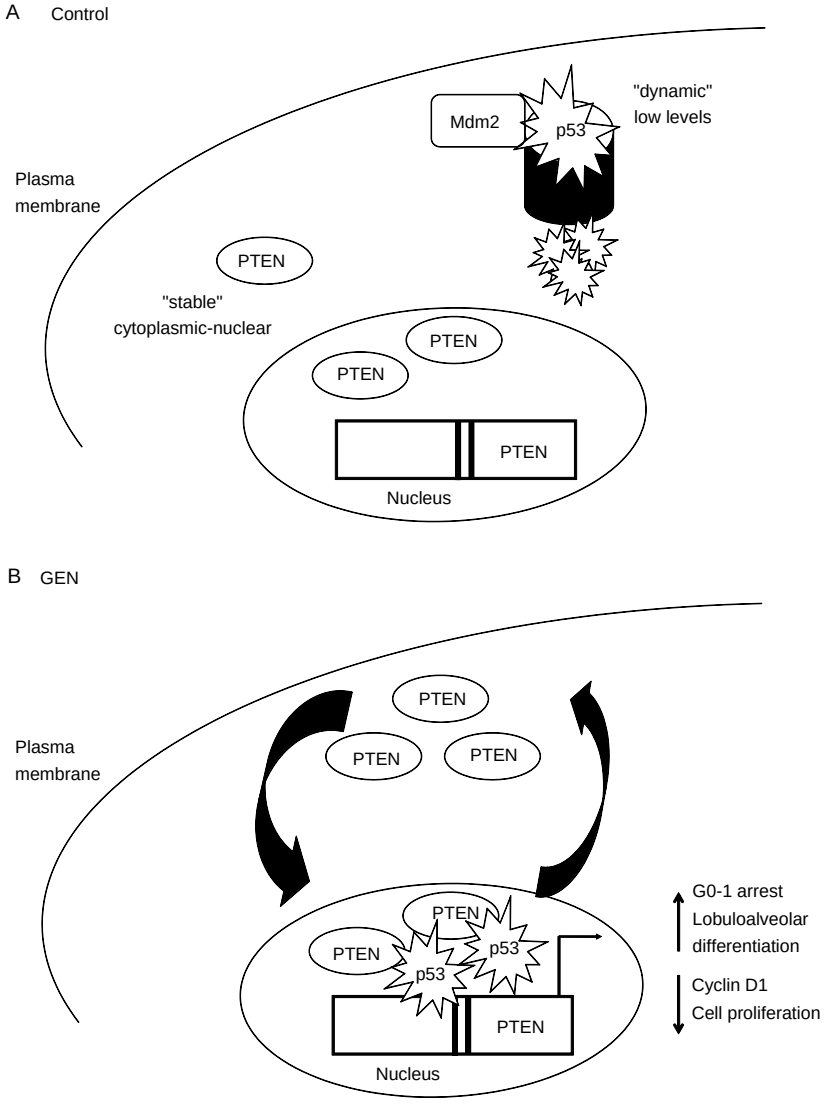

Figure 1. In the presence of GEN, the nuclear pool of PTEN is increased leading to increased levels of nuclear p53, promotion of PTEN/p53 complex formation, increased recruitment of the PTEN/p53 complex, and hence activation of PTEN transcription. A functional outcome of increased nuclear PTEN is the reduction in the expression of pro-proliferative genes cyclin D1 and pleiotrophin, resulting in G0-1 arrest and leading to early lobuloalveolar differentiation (Adapted from Rahal and Simmen, 2010).

To address Aim 2, I investigated SPI effects relative to the control CAS diet, on mammary tumor development in MMTV-Wnt1-Transgenic (Wnt1-Tg) female mice and on the mammary stem cell (SC) population in pre-neoplastic Wnt1-Tg female mice. From gestation day 4 (GD 4), dams were fed the control CAS diet, composed of casein as the sole protein source. Female Wnt1-Tg pups continued on CAS diet until weaned at PND 21. At weaning, female Wnt1-Tg mice were assigned to different semipurified isocaloric diets according to the American Institute of Nutrition (AIN-93G) formulation. The diets are 1) CAS diet, casein is the sole protein source, 2) SPI diet, soy protein isolate as sole protein source containing 430 mg of total isoflavones/kg diet, including 276 mg/kg genistein and 132 mg/kg daidzein, and 3) GEN diet, made of casein with addition of aglycone GEN. Mice were fed these diets throughout the study. Mammary tumor latency, incidence and multiplicity were carefully observed and recorded. For stem cell analysis, MECs were isolated from preneoplastic PND75, virgin Wnt1-Tg female mice fed CAS or SPI and used for FACS analysis, transplantation assays, mammosphere and colony formation assays. Data generated from Aim 2 has been incorporated into two manuscripts (*in preparation*) describing the effect of dietary SPI or GEN, relative to control CAS diet, on regulation of MaSC/CSCs and tumor formation in Wnt1-Tg mice (outlined below).

Manuscript 1: Dietary Regulation of Mammary Stem/Progenitor Cells is Associated with Protection Against Wnt1-driven Mammary Tumorigenesis*[*, ǂ, #]

* This work is currently being reviewed in *Stem Cell Research*
ǂ *Poster Contest Finalist* - Era of Hope Conference, August 2-5, 2011, Orlando, Florida.
\# *Poster Discussion* - Selected Abstract at the 33rd Annual San Antonio Breast Cancer Symposium, December 8-12, 2010, San Antonio, Texas.

Dietary SPI protects against spontaneous mammary tumor formation in Wnt1-Tg mice

Epidemiological studies suggest an inverse association between high consumption of soy foods and breast cancer risk (1). We previously showed that lifetime dietary intake of SPI protects against chemical-induced mammary tumorigenesis in rats (2, 4). To investigate the effect of dietary SPI on spontaneous mammary tumor formation, we used Wnt1-Tg female mice as a model for human breast cancer (7). As shown in Figure 2A, greater than one-half (53.3%) of Wnt1-Tg mice fed SPI beginning at post-weaning were tumor-free during the 8 months of the study. In contrast, the majority of CAS-fed mice (77%) harboring the Wnt1 transgene developed mammary tumors. Interestingly, the decrease in tumor incidence with dietary SPI was accompanied by shorter tumor latency (SPI vs. CAS: 4.65 vs. 5.88 months, $P = 0.023$) (Fig. 2B). Tumor growth (within two weeks after initial detection) and tumor weights at sacrifice were similar in both groups (Fig. 2C and 2D). Dietary SPI decreased body weight of Wnt1-Tg females enrolled in the tumor study by PND77 when compared to CAS-fed mice (Fig. 2E). Transcript levels of Wnt1 in mammary tumors did not differ between diet groups (Fig. 2F). Histopathologic features of mammary tumors showed predominance of solid carcinoma, which was greater for SPI- than CAS-fed mice (Table 1) (17).

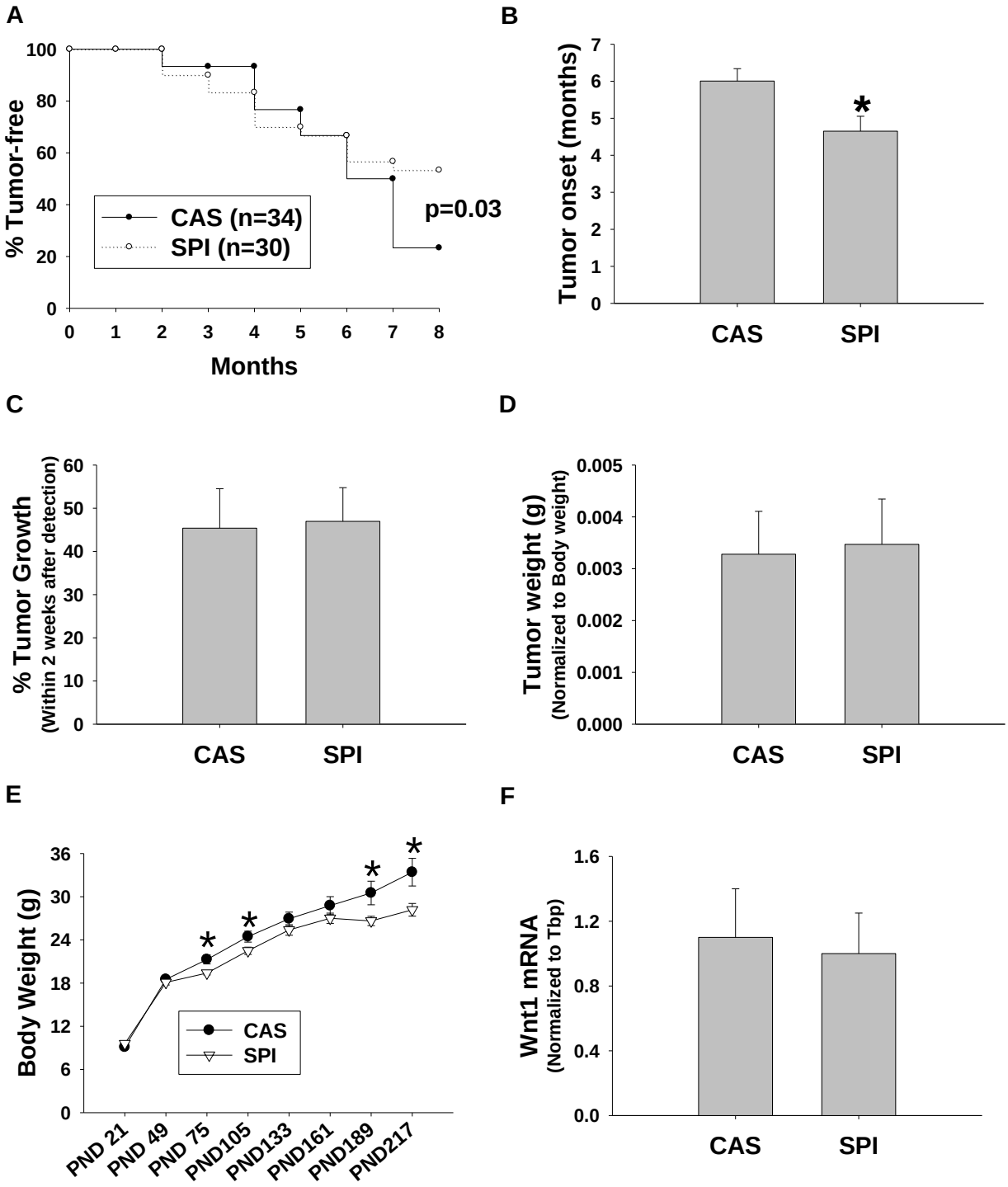

Figure 2. Soy protein isolate (SPI) protects against Wnt1-induced breast cancer. (A) Percentage of tumor-free Wnt-1Tg female mice fed casein (CAS; n=34) or SPI (n=30). (B) Tumor latency for Wnt1-Tg mice that developed tumors. * P<0.05 for SPI relative to CAS. SPI diet had no effect on tumor growth (C) or weight (D). (E) Body weights of Wnt1-Tg mice enrolled in the mammary tumor study were recorded monthly from weaning (PND21) until study conclusion (up to 8 months). * P < 0.05 relative to CAS. (F) *Wnt1* mRNA levels were quantified by QPCR in tumors from Wnt1-Tg mice exposed CAS or SPI diets. *Tbp* was used as a normalizing control.

Table 1. Histopathologic features of mammary tumors

Designation	% Incidence	
	CAS[a]	SPI[b]
Solid carcinoma	50	70
Papillary carcinoma	25	15
Cribriform carcinoma	5	
Adenosquamous carcinoma	5	5
Glandular carcinoma		5
Mammary carcinoma in situ	5	
Lymph node hyperplasia	5	5
Lymph node with metastasis	5	

[a, b] n = 20

SPI downregulates MaSC-enriched and cancer stem cell populations in preneoplastic mammary glands of Wnt1-Tg mice

To determine whether the inhibitory effects of dietary SPI on mammary tumor incidence (Fig. 2A) are associated with regulation of MaSC population, the percentage of MaSCs in freshly isolated mammary epithelial cells (MECs) from preneoplastic mammary glands of virgin PND75 Wnt1-Tg mice of the two diet groups, was quantified by FACS, based on the expression of well-characterized mouse MaSC markers CD29 (β1 integrin) and CD24 (heat stable antigen) within the Lineage negative (Lin^-) population ($CD45^-$, $TER119^-$, $CD31^-$). The MaSC-enriched subpopulation ($CD29^{hi}CD24^+$) was decreased by 50% ($P < 0.001$) in preneoplastic mammary glands of Wnt1-Tg mice fed SPI relative to those fed CAS (Fig. 3A and 3B). The latter occurred in the absence of similar effects on luminal ($CD29^{lo}CD24^+$) population (Fig. 3C). The basal ($CD29^{hi}CD24^+$), MaSC-enriched population was expanded (~ 3-fold) in mammary glands of PND75 Wnt1-Tg mice compared to WT counterparts (4.8 ± 1.22 vs. 14.02 ± 0.84%, WT vs. Wnt1-Tg; $P < 0.001$) (Fig. 3D) similar to previous studies (10, 13), which in turn, could facilitate conversion into CSCs (11). Further fractionation of the MaSC-enriched subpopulation ($CD29^{hi}CD24^+$) using the surface marker Thy1, shown previously to identify the CSC population (Thy1+CD24+) in the Wnt1-Tg model (11), resulted in the isolation of a very small percentage (~1%) of the total bulk of epithelial cells in the CAS-fed group; this was decreased (by 2-fold; $P = 0.012$) in mammary glands of SPI-fed Wnt1-Tg mice (Fig. 3E). Diet did not alter the MEC yield ($2.25 \pm 0.67 \times 10^6$ vs. $2.37 \pm 0.4 \times 10^6$ cells/gram of mammary tissue; CAS vs. SPI, respectively).

Transplantation of MECs into the cleared fat pads of recipient mice results in the reconstitution of a functional mammary gland, which is indicative of MaSC activity (18). Previous studies showed that MECs from preneoplastic Wnt1-Tg mice exhibited high *in vivo* repopulating activity upon transplantation, suggesting that there is an expansion of MaSCs in the mammary glands (10). Thus, transplantation assays were performed to determine whether the SP1 diet affects mammary gland repopulation. Ten thousand MECs isolated from PND75 Wnt1-Tg mice fed either CAS or SPI were transplanted into three-week old syngeneic WT recipient mice. As shown in Figure 3F, MECs from both CAS- and SPI-fed Wnt1-Tg mice generated a complete mammary epithelial tree after transplantation. Interestingly, while 6 of 8 MEC

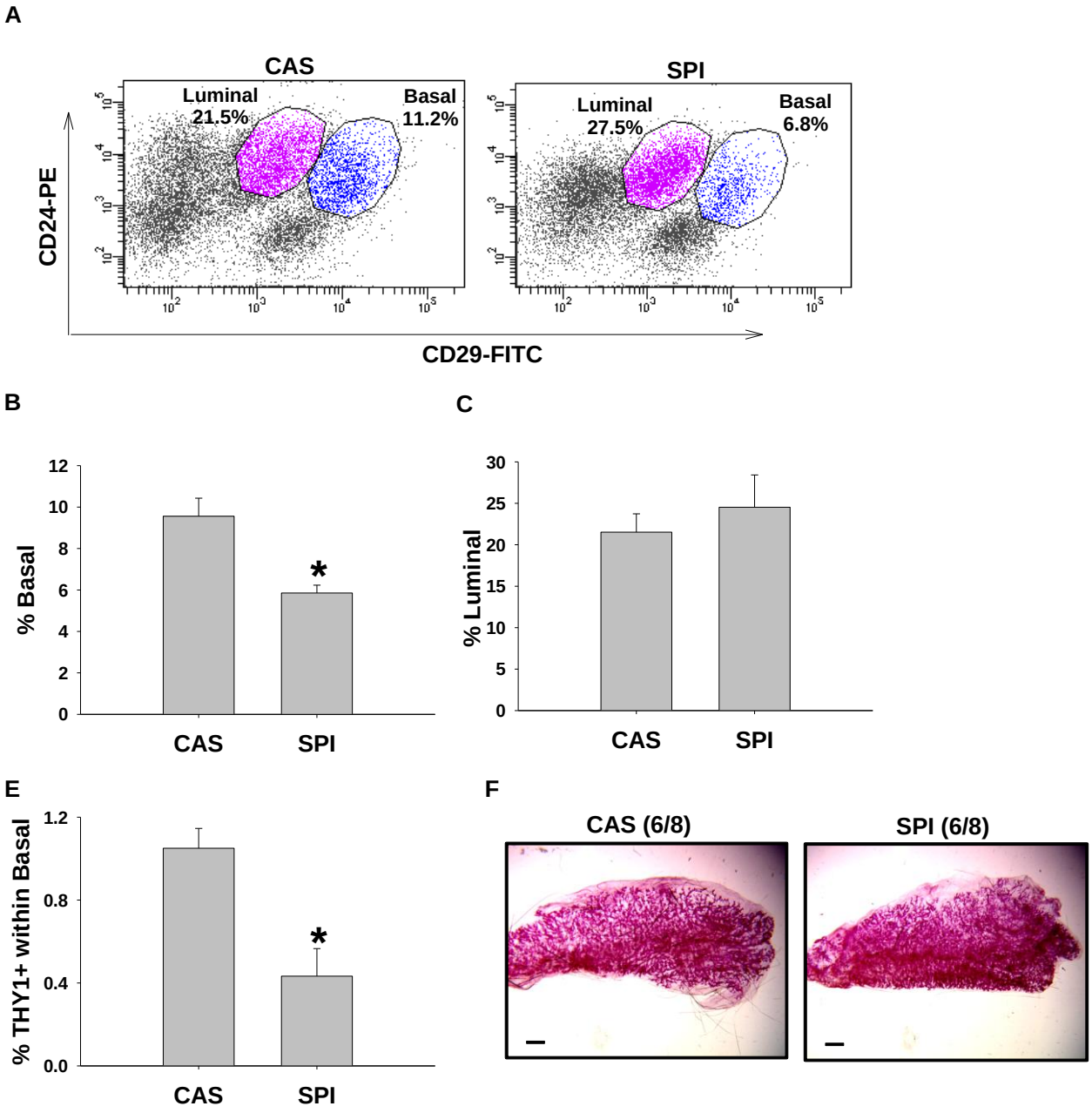

Figure 3. Dietary SPI decreases MaSC-enriched and CSC populations in preneoplastic mammary glands of Wnt1-Tg mice. (A) Representative FACS plot of MECs from preneoplastic PND75 Wnt1-Tg mice fed CAS (*Left*) or SPI (*Right*) based on the expression of mouse MaSC markers CD29 and CD24 within the lineage negative ($CD45^-$, $TER119^-$, and $CD31^-$) population after exclusion of dead cells ($DAPI^+$). (B) SPI diet reduced MaSC-enriched ($CD29^{hi}CD24^+$) population. (C) Diet had no effect on luminal ($CD29^{lo}CD24^+$) population. (D) Basal, MaSC-enriched ($CD29^{hi}CD24^+$) population is expanded (~ 3-fold) in mammary glands of PND75 Wnt1-Tg CAS-fed mice compared to WT counterparts. n=6 per genotype; $P < 0.001$ relative to WT. (E) SPI reduced the cancer stem cell (Thy1+CD24+) population in preneoplastic mammary tissue of Wnt1-Tg mice. Data shown are from at least 5-7 independent experiments. * $P < 0.05$ relative to CAS. (F) Whole mounts depicting positive mammary outgrowths from transplantation of freshly isolated MECs (10,000 cells) from preneoplastic mammary glands of Wnt1-Tg mice fed CAS (6/8 outgrowths) or SPI (2/8 outgrowths). Scale bar, 1000 µm (for outgrowth, *left panels*) and 500 µm (for no outgrowth, *right panels*).

transplants from CAS-fed Wnt1-Tg mice generated outgrowths in WT recipient mice, only 2 of 8 outgrowths were observed from MECs of SPI-fed mice (90-100% fat pad filled). These findings suggest that exposure to the SPI diet in a neoplastic environment such as with Wnt1 over-expression, limits the activity of the MaSC-enriched population ($CD29^{hi}CD24^{+}$).

MECs from SPI-fed Wnt1-Tg mice exhibit lower mammosphere forming efficiency

To further evaluate dietary effects on mammary stem and progenitor cells in MECs isolated from preneoplastic Wnt1-Tg mice, the mammosphere formation assay, based on the ability of a small population of mammary stem/progenitor cells to grow in suspension as spheres (19), was used. With this assay, mammospheres formed from primary passage were largely comprised of restricted progenitor (basal or luminal epithelial) cells while those from additional passages represented cells enriched for early, bipotent progenitors and stem-like cells (20). As shown in Figure 4A, MECs from CAS- and SPI-fed Wnt1-Tg mice formed similar numbers of mammospheres on primary passage (Passage 1; P1), suggesting that diet had a minimal effect on the restricted progenitor population. Passage of primary spheres resulted in lower numbers of mammospheres formed at Passage 2 (P2) for MECs of SPI-fed than CAS-fed Wnt1-Tg mice (Fig. 4B), suggesting dietary regulation of early mammary progenitors and stem-like cells.

Luminal progenitor population is refractory to dietary SPI exposure

The mouse mammary luminal population ($CD29^{lo}CD24^{hi}$) is composed of both luminal progenitors and differentiated luminal cells (18). Luminal progenitors are enriched for colony-forming activity in Matrigel (21). Further, Vaillant and colleagues (13) have identified this epithelial sub-population from mammary tumors of Wnt1-Tg mice to exhibit tumor-initiating activity. To determine whether dietary regulation of the mammary luminal progenitor subpopulation is associated with mammary tumor protection in Wnt1-Tg mice (Fig. 2A), we measured the ability of MECs to form colonies in Matrigel (Fig. 4C and 4D). MECs from preneoplastic PND75 Wnt1-Tg mice fed CAS or SPI formed similar number of colonies in Matrigel culture (225 ± 15.15 vs. 231 ± 8.69; per 5000 cells) (Fig. 4D). These results suggest that the mammary tumor protective effects of SPI may not involve regulation of luminal progenitors.

Dietary SPI exposure decreases systemic levels of progesterone and estradiol

While both human and mouse MaSCs lack the expression of estrogen receptor (ER) and progesterone receptor (PR) (21, 22), MaSC activity is under hormonal regulation via paracrine signaling from the luminal compartment (23, 24). To determine whether the observed dietary SPI effects on the MaSC population are correlated with systemic changes in the hormonal milieu, serum levels of estradiol-17β and progesterone were measured for PND75 Wnt1-Tg mice used in the stem cell analysis (Fig. 3). Levels of progesterone were lower (by 2.2-fold) in SPI-fed than in CAS-fed Wnt1-Tg mice (12.39 ± 2.79 vs. 5.66 ± 1.12 ng/ml; $P = 0.06$). Similarly, serum estradiol-17β concentrations were 2.6-fold lower in SPI-fed than the CAS-fed group (34.03 ± 1.97 vs. 13.06 ± 0.76 pg/ml; $P < 0.001$). Thus, SPI effects on MaSC/progenitor populations may involve, in part, changes in systemic steroid hormone levels.

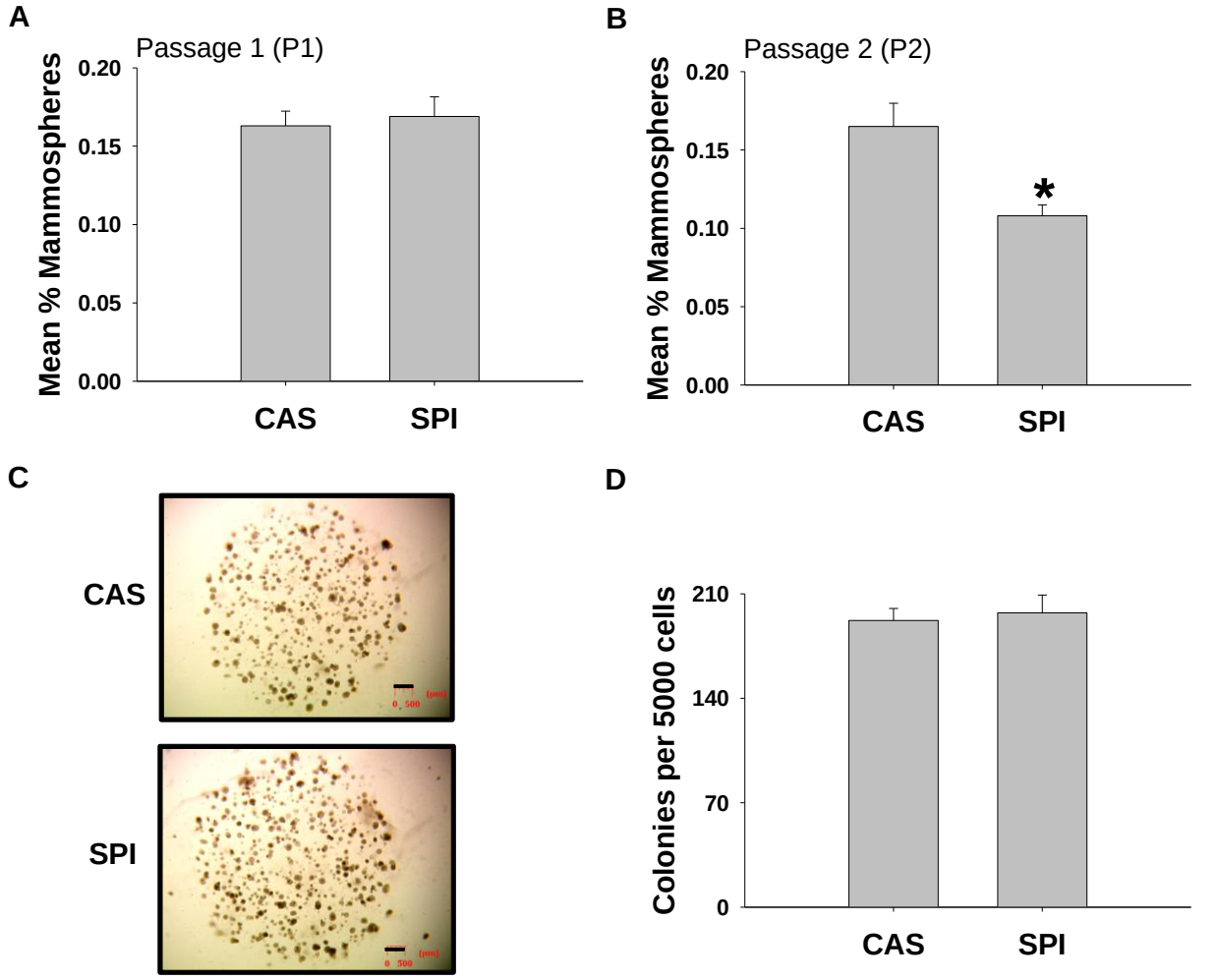

Figure 4. MECs from SPI-fed Wnt1-Tg mice have lower mammosphere forming efficiency. (A) MECs from CAS- and SPI-fed PND75 Wnt1-Tg mice had similar primary mammosphere formation efficiency, measured as mammosphere-forming units (MFU). (B) MECs from SPI-fed Wnt1-Tg mice have lower ability to form secondary mammospheres. Data represent the mean % MFUs ± SEM of seven independent experiments (n=16 per experiment); * $P < 0.05$ relative to CAS. (C) Representative images depicting colony-forming ability of MECs from CAS and SPI-fed PND75 Wnt1-Tg mice. Scale bar, 500 µm. (D) Diet had no effect on colony forming ability of MECs from Wnt1-Tg mice. Histograms show mean ± SEM of five independent experiments (n= 6-12 replicates per experiment/diet).

MaSC-enriched population from SPI-fed Wnt1-Tg mice exhibits down-regulated expression of genes associated with poor breast cancer outcome

In order to identify novel genes and networks that mediate dietary regulation of MaSCs and breast CSCs (Fig. 3), we performed microarray analysis on FACS-sorted MaSC-enriched populations ($CD29^{hi}CD24^{+}$) from mammary glands of preneoplastic PND75 Wnt1-Tg mice fed CAS or SPI. Unsupervised hierarchical clustering of gene expression indicated that CAS samples grouped separately from those of the SPI group (data not shown). A total of 907 genes were significantly regulated by at least 1.3-fold with dietary intake of SPI. Of these, 297 were upregulated (Table 2) and 610 were downregulated (Table 3) in MaSCs with SPI diet. The list for SPI upregulated genes included candidate tumor suppressor genes methylated or lost in

Table 2 List of upregulated genes by SPI in the MaSC-enriched population from PND75 Wnt1-Tg mice.

Upregulated in SPI							
Abhd10	Cdan1	Fam3c	Hist1h3i	Lmnb1	Padi3	Sag	Trp63
Accn1	Cenpa	Fam49b	Hist2h3c1	Lnx2	Pappa	Sema3c	Tsc22d3
Acly	Cfp	Fam65b	Hist1h3h	Mir1949	Parm1	Sema4c	Ttc26
Acot6	Chac1	Fam82a1	Hist1h3g	Luc7l2	Pcp4	Sema6d	Tubgcp5
Adamts9	Chrm3	Fen1	Hist2h3b	Lrrc4	Pde4dip	Serpinb6b	Twist1
Adcy9	Cldn8	Fgfr2	Hist1h3f	Lrrc4c	Pdk4	Serpinb9	Ugt1a1
Adipoq	Cml1	Fhod3	Hist1h3e	Lrrc8c	Per1	Serpine1	Ugt1a2
Adm	Cml5	Flnc	Hist1h3d	Maob	Per2	Sipa1l2	Ugt1a5
Adra2a	Cobll1	Fosb	Hist1h3c	Map2k6	Pfas	Slc14a1	Ugt1a6a
Aff2	Cpa6	Gal	Hist1h3b	Map3k5	Pgm5	Slc15a2	Ugt1a6b
Ak1	Cpt1a	Galntl4	Hist1h4c	Matn2	Pik3r4	Slc2a13	Ugt1a7c
Akap11	Crybb1	Gas2l3	Hist1h4h	Mkx	Plcb1	Slc7a5	Ugt1a9/
Ang	Ctnnal1	Gfra1	Hist1h4i	Mlf1	Plcb4	Smarcal1	Ugt1a10
Angptl4	Cttnbp2	Ggnbp1	Hist1h4j	Mmd	Plin4	Snord89	Vcan
Ankrd56	Cxcr4	Glp1r	Hist1h4k	Mmp12	Plk3	Spinlw1	Vwce
Ap1s3	Cyp24a1	Glrb	Hist1h4m	Ms4a6b	Ppp1r14d	Spock1	Was
Aqp9	Cyp2c37	Gnai1	Hist1h4a	Mtap1b	Prdm1	Spock2	Wisp1
Arsj	Dctd	Gpr50	Hist1h4b	Mtap7d2	Prickle2	Sprr2a1	Wnk2
Art4	Dennd2c	Gprin3	Hmgcll1	Mthfd2	Prkd1	Sprr2a2	Zbtb16
Atic	Dgat2	Grik4	Hsdl2	Mtmr7	Prr5l	Sprr2a3	Zdbf2
Bat1a	Dido1	Gsta3	Hspe1	Myst1	Psat1	Sspn	Zfc3h1
Bcar3	Diras2	Gtf3c3	Hyal1	Nap1l2	Psd2	Stk17b	Zfp12
Bcat1	Dnahc17	Gtsf1l	Il17rb	Nap1l5	Psph	Tbc1d1	Zfp395
Bcl6	Dnase1	H2-Q5	Il20	Nat6	Ptpre	Tg	Zfp655
Bdnf	Ear1	Hadha	Il24	Nceh1	Ptx3	Tgfbr3	Zfp770
Bmp8a	Ear2	Hadhb	Ints2	Ncf4	Pxk	Them5	Zfp874a
Bmpr1b	Ear3	Hal	Itga8	Ndrg2	Pygb	Thrsp	Zmat4
Bnc1	Ear12	Hao1	Kbtbd7	Ndufaf1	Qsox2	Tiam2	Zmym6
C1qc	Efhb	Hcn3	Kcnmb2	Nfatc2	Rab39b	Tigd2	Zscan18
C2cd4b	Egfl6	Hhip	Kidins220	Nhsl1	Rasl11b	Tmem14a	Zswim7
Capn3	Egr4	Hist1h1e	Klf11	Nme6	Ren1	Tmem229b	Zwint
Casq2	Eid3	Hist1h2bc	Klk1b16	Nrg1	Ren2	Tmem39a	
Cbln1	Emb	Hist1h2be	Krt15	Ntrk3	Ret	Tmem88b	
Ccdc62	Enc1	Hist1h2bp	Krt73	Nudt5	Rgs12	Tmtc1	
Ccnf	Entpd5	Hist1h2bm	Krtap22-2	Obox1	Rnd3	Top1mt	
Ccno	Ets1	Hist1h2bl	Lancl3	Obox2	Rorc	Tppp	
Ccrl1	Fam13a	Hist1h3a	Lgals4	Orc1	Rrm2	Trim32	
Cd5l	Fam13c	Hist2h3c2-ps	Lgals7	Oxtr	Rtn1	Trp53inp1	

Table 3 List of downregulated genes by SPI in the MaSC-enriched population from PND75 Wnt1-Tg mice.

Downregulated in SPI							
Aass	Avpi1	Ccl21b	Cmpk1	Dct	Esr1	Gimap6	Homer2
Abca8a	B2m	Ccl21c	Cnfn	Ddr2	Esrp2	Gipc1	Hopx
Abcc9	Barx2	Ccl3	Col1a1	Ddx60	Esrrg	Gjb2	Hpse
Abcg1	Basp1	Ccl5	Col1a2	Dhcr7	Esyt3	Gjb6	Hsd11b1
Abi3bp	Bbox1	Ccl8	Col3a1	Dhx32	Ezh2	Gjc1	Htra3
Acsl1	Bcam	Ccnd1	Col5a2	Dmkn	Fabp4	Gltp	Ide
Adamts12	Bckdhb	Cd274	Col5a3	Dpp4	Fam108c	Glycam1	Idi1
Adamts4	Bcl2l15	Cd34	Col6a1	Dpt	Fam46b	Csprs	Ifi203
Adamts5	Bcl3	Cd59a	Col6a2	Dsc2	Fam59a	Gna14	Ifi27l2a
Adamts7	Bdh1	Cd82	Col6a3	Dsg1a	Fap	Gnb4	Ifi35
Adcy7	Bend7	Cdh11	Colec12	Dtx3l	Fbln5	Gprc5a	Ifi47
Add3	Bex1	Cdh5	Cpa3	Duox1	Fbp1	Gramd4	Ifih1
Adh1	Bglap	Cdkn2b	Cpxm1	Duoxa1	Fbxo6	Grasp	Ifit1
Afap1l2	Bglap2	Cdo1	Crabp1	Ebf1	Fcer1a	Gsn	Ifit2
Akr1c14	Bglap-rs1	Ceacam1	Crabp2	Ecscr	Fcgr2b	Gsta4	Ifit3
Aldh1a1	Bicc1	Ceacam2	Crip1	Ednrb	Fdft1	Gstt1	Igf1
Aldh1a3	Birc5	Cel	Crispld1	Efcab4a	Fgf14	Gstt2	Igfbp6
Aldh3a1	Blnk	Cerk	Cryba4	Efemp1	Fgfbp1	Gulp1	Igfbp7
Alox15	Bmper	Cfh	Csf3	Egflam	Fgl2	Gvin1	Igh-3
Angpt2	Bnipl	Chad	Csn1s1	Ehf	Figf	H2-D1	Igh-6
Anxa1	Bspry	Chchd10	Csn1s2a	Eif2ak2	Flrt2	H2-K1	Igh-VJ558
Anxa2	Bst1	Chi3l1	Csrp2	Eif2ak3	Fmn1	H2-L	Ighg
Anxa3	Bst2	Chl1	Cthrc1	Eif4e3	Fmod	H2-Q2	Ighg1
Anxa8	C1qtnf2	Chst1	Ctsh	Elf3	Fn1	H2-Q6	Igj
Aoc3	C1ra	Chst12	Ctsk	Elovl7	Foxc1	H2-Q7	Igtp
Aplnr	C1rb	Cilp	Cwh43	Emilin2	Frg1	H2-T3	Iigp1
Apoc1	C1s	Ckmt1	Cxcl10	Emp2	Frk	H2-T3-like	Il18
Apol7a	C2	Clca1	Cxcl11	Emp3	Fscn1	H2-T9	Il18r1
Apol7c	Cfb	Clca2	Cxcl13	Enah	Fut9	H2-T10	Il1a
Apold1	Calcrl	Clcn3	Cxcl5	Endod1	Fxyd3	H2-T18	Il1r1
Aqp1	Calml3	Cldn10	Cxcl9	Enpep	Fxyd6	H2-T22	Il1rn
Arap2	Cap1	Cldn7	Cyb561	Enpp2	Gabrp	H2-T23	Il6
Arg1	Car2	Clec11a	Cygb	Enpp3	Gbp10	Has1	Inhbb
Aspa	Card11	Clec3b	Cyp1b1	Epb4.1l4a	Gbp2	Hc	Inmt
Aspn	Ccdc80	Clic3	Cyp4f39	Epha3	Gbp3	Herc6	Irf2
Asprv1	Ccl11	Clip4	Cytip	Eppk1	Gbp6	Hes1	Irf9
Atp10a	Ccl19	Clu	Cyyr1	Epyc	Gdf10	Hexa	Irgm1
Atp1b1	Ccl21a	Cma1	Daf2	Erbb3	Gdpd1	Homer1	Irgm2

Downregulated in SPI cont.							
Isg15	Loxl4	Mustn1	Pde7b	Rab25	Serpina1d	Sprr1a	Tnc
Islr	Lpar1	Mxd1	Pdgfra	Ralbp1	Serpina1e	Srgn	Tnf
Itgb6	Lpl	Mxra8	Pdgfrb	Ramp2	Serpina3g	Srpx2	Tnfaip2
Itgbl1	Lrrc26	Naalad2	Pdzk1ip1	Rarres2	Serpinb1a	Ssfa2	Tnfaip3
Itih5	Lrrfip1	Naaladl2	Pea15a	Rbp7	Serpinf1	St8sia6	Tnfaip6
Itm2a	Ltbp1	Nfe2l3	Pgd	Rgs4	Serping1	Stag3	Tnnt2
Jup	Ltf	Nfkbia	Pi16	Rgs5	Sfrp2	Stap2	Trim2
Kcne4	Lum	Nid1	Pid1	Rhov	Sh2d4a	Stard4	Trim30a
Kcnn4	Ly6a	Nipal1	Pigr	Rnase1	Sh3kbp1	Stat1	Trim30d
Kcnq1	Ly6d	Nipal2	Pik3ap1	Rnf125	Sh3yl1	Steap3	Trim68
Kctd14	Ly96	Nkd2	Pkp2	Rnf152	Shc4	Stra6	Trpm4
Kif26a	Lypd3	Notch2	Pla1a	Rsad2	Shisa4	Sulf2	Trpv6
Kit	Lypd6b	Notch3	Pla2g16	Rtp4	Sigirr	Sult1a1	Tspan33
Klk11	Mal	Npas2	Plcg2	Runx3	Slc1a1	Susd2	Tspo
Krt18	Mal2	Nr2f1	Plekha7	S100a14	Slc28a3	Svep1	Tubb2a-ps2
Krt19	Mall	Nrn1	Plekhg1	S100a4	Slc40a1	Syngr2	Tubb2b
Krt4	Map3k1	Nrp1	Plscr1	S100a6	Slc41a2	Tacstd2	Tuft1
Krt6a	Mapk13	Ntn1	Plscr2	S1pr3	Slc43a2	Tagln2	Ugcg
Krt6b	Marveld2	Nts	Plvap	Saa1	Slc44a3	Tap1	Usp18
Krt7	Marveld3	Nupr1	Pmm1	Saa2	Slc5a1	Tgtp1	Vat1
Krt79	Mcc	Oas1a	Pnpla2	Saa3	Slc5a9	Tgtp2	Vcam1
Krt80	Mcpt4	Oasl1	Pof1b	Samhd1	Slc9a3r1	Thbd	Vtn
L1cam	Meox2	Oasl2	Postn	Sbsn	Slfn8	Thbs2	Vwf
Lad1	Mfap4	Obfc1	Ppp1r9a	Scara3	Slit2	Thy1	Wap
Lama2	Mfap5	Ocln	Prkch	Scara5	Slit3	Tie1	Wbscr27
Lass3	Mfsd6	Ogn	Prlr	Scarb1	Slk	Timp3	Wfdc2
Ldb2	Mgam	Olr1	Prrt2	Scd1	Smagp	Tlr4	Wnt5b
Lgals1	Mical2	Ormdl2	Prss8	Scd2	Smoc2	Tmc4	Wwc1
Lgals3	Mmp13	Osbpl3	Psapl1	Scel	Sorbs2	Tmem140	Xaf1
Lgals3bp	Mmp19	P4ha2	Psmb8	Scnn1a	Sox17	Tmem176a	Zbp1
Lif	Mmp3	Pamr1	Pstpip2	Sele	Sox7	Tmem30b	Zbtb7c
Lin7a	Mmrn2	Parp14	Ptplad2	Selp	Sparcl1	Tmem87a	Zeb1
Lipg	Mnda	Parp9	Ptprb	Sept4	Spats2l	Tmod3	Zeb2
Lmo2	Mpped2	Pbxip1	Ptprz1	Sept5	Spink5	Tmprss11a	Zfhx3
Lmo7	Mras	Pcdh1	Pvrl4	Serpina10	Spint1	Tmprss11d	Zfp458
Lox	Mrc2	Pcdh17	Pygl	Serpina1a	Spns2	Tmprss11e	Zfp472
Loxl1	Ms4a4d	Pcolce	Qpct	Serpina1b	Spon1	Tmprss13	Zfp600
Loxl2	Mtm1	Pde1c	Rab11fip1	Serpina1c	Spp1	Tmprss4	Zfp605
							Zfp874b
							Zfp97

several cancers, including breast cancer, such as basonuclin 1 (*Bnc1*); catenin alpha-like-1 (*Ctnnal1*); cytochrome P450, family 24, subfamily a, polypeptide 1 (*Cyp24a1*); FBJ osteosarcoma oncogene B (*Fosb*); krüppel-like factor 11 (*Klf11*); pyruvate dehydrogenase kinase, isoenzyme 4 (*Pdk4*); and WNK lysine deficient protein kinase 2 (*Wnk2*).

Dietary SPI attenuated the expression of numerous genes previously shown to be enriched in MaSCs and breast CSCs and which are associated with poor clinical outcome; these include aldehyde dehydrogenase 1 (*Aldh1*); interleukin 6 (*Il6*); notch gene homolog 2 (*Notch2*); and thymus cell antigen 1 (*Thy1*). Beta 2-microglobulin (*B2m*), a protein that is upregulated in multidrug-resistant tumor cells (25), was also decreased in MaSC from SPI-fed mice. SPI also downregulated gene expression of signal transducer and activator of transcription 1 (*Stat1*), recently shown to mediate radiation resistance of human breast cancer stem cells ($CD44^{+}/CD24^{-/low}$) (26). Of significance is the noted reduction by SPI of expression of several genes involved in breast cancer migration, epithelial-to-mesenchymal transition (EMT), and/or metastasis in MaSC-enriched population; these include bone marrow stromal protein 2 (*Bst2*), fatty acid–binding protein 4 (*Fabp4*, also known as *aP2*), alpha-1 type I collagen (*Col-1*), endonuclease domain containing 1 (*Endod1*), enhancer of zeste homolog 2 (*Ezh2*), fibulin 5 (*Fbln5*), periostin (*Postn*), L1 cell adhesion molecule (*L1cam*), toll-like receptor 4 (*Tlr4*), tenascin c (*Tnc*), E-selectin (*Sele*), P-selectin (*Selp*), zinc finger E-box binding homeobox 1 (*Zeb1*), and *Zeb2*.

Inflammatory response, chemokine, and cytokine network genes are targets of dietary SPI in MaSC-enriched population

We performed gene set enrichment analysis (GSEA) to gain insight into the biological processes and functions that are altered by diet and which may have a role in dietary regulation of MaSC self-renewal and mammary tumorigenesis. As shown in Table 4, the major gene functions regulated by SPI (enriched or diminished with SPI) were related to immune-(inflammation, chemokine activity, cytokine activity), oxidative stress, protein metabolism, and tyrosine kinase activities. The heat maps of inflammatory genes that constitute cytokine networks involved in the regulation of breast CSCs (27) and which are downregulated with dietary SPI are shown in Figure 5. Genes in these categories include those for proinflammatory cytokines tumor necrosis factor (*Tnf*) and interleukin 6 (*Il6*), and for the chemokine (C-C motif) ligands *Ccl5*, *Ccl7*, and chemokine (C-X-C motif) ligand *Cxcl5*. Other gene networks influenced by SPI diet include those underlying cell proliferation (Fig. 5C) and protein metabolism (Fig.5E).

Ingenuity Pathway Analysis (IPA) on the 907 annotated genes regulated by SPI ($\geq$ 1.3-fold) in MaSCs from preneoplastic Wnt1-Tg mice revealed the top gene networks, functional processes, and canonical pathways influenced by dietary SPI, relative to CAS. These include those involved in organ development, cancer, reproductive system disease, metabolic disorders, and cell-cell signaling. The overlapping networks of genes involved in MaSC and/or breast CSC self-renewal (*Aldh, B2m, Il6, Notch2, Stat1, Thy1*), inflammatory response/chemokine signaling (*Ccl8, Ccl11, Ccl21, Cxcl6, Cxcl10, Cxcl11, Tlr, Tnf*), and migration/EMT/metastasis (*Bst2, Ezh2, Selp, Tnc, Zeb1*) that are impacted by dietary SPI in MaSC-enriched (as well as CSC) populations are summarized in Figure 6A. Altered expression by diet of a subset of key genes involved in breast cancer stem cell survival (*Il6, Stat1*) (26, 28), inflammation/chemokine signaling (*Il6, Ccl8*) (27-31), and metastasis (*Tnc*) (32) were confirmed by QPCR (Fig. 6B).

Serum levels of IL6 are consistently upregulated in breast cancer patients (33), suggesting elevated serum IL6 as a negative prognostic marker in breast cancer. As shown in Figure 6C, serum levels of IL6 were virtually abolished in preneoplastic Wnt1-Tg mice fed SPI, relative to CAS-fed mice. Results suggest that dietary SPI negatively regulates local (MaSC-enriched population) and systemic IL6 production. Based on the signaling pathways reported to be regulated by the cytokines in this gene signature, we propose a model in which IL6 may function as central mediator of dietary SPI effects (Fig. 6D). Taken together, results show dietary regulation of mammary stem/progenitor cells *in vivo* to inhibit tumor susceptibility and identify diet-regulated, stem cell-associated genes for application in breast cancer therapy.

Table 4. Summary of GSEA with FDR no more than 0.25

Gene set	FDR
Enriched in SPI	
Tube development	0.151
Transferase activity transferring groups other than amino acyl groups	0.230
Diminished in SPI	
Chemokine signaling	< 0.001
Cytokine activity	< 0.001
Protein tyrosine kinase activity	< 0.001
Inflammatory response	0.004
Negative regulation of protein metabolic process	0.011
Immune response	0.079
Tissue development	0.079
Cytokine metabolic process	0.093
Regulation of cell proliferation	0.094
Cytokine biosynthetic process	0.103
Ras GTPase activator activity	0.109
Lipid binding	0.122
Receptor signaling protein activity	0.124
Response to oxidative stress	0.170
Cell cell signaling	0.169
Interleukin receptor activity	0.241
Oxidoreductase activity	0.241

FDR, false discovery rate

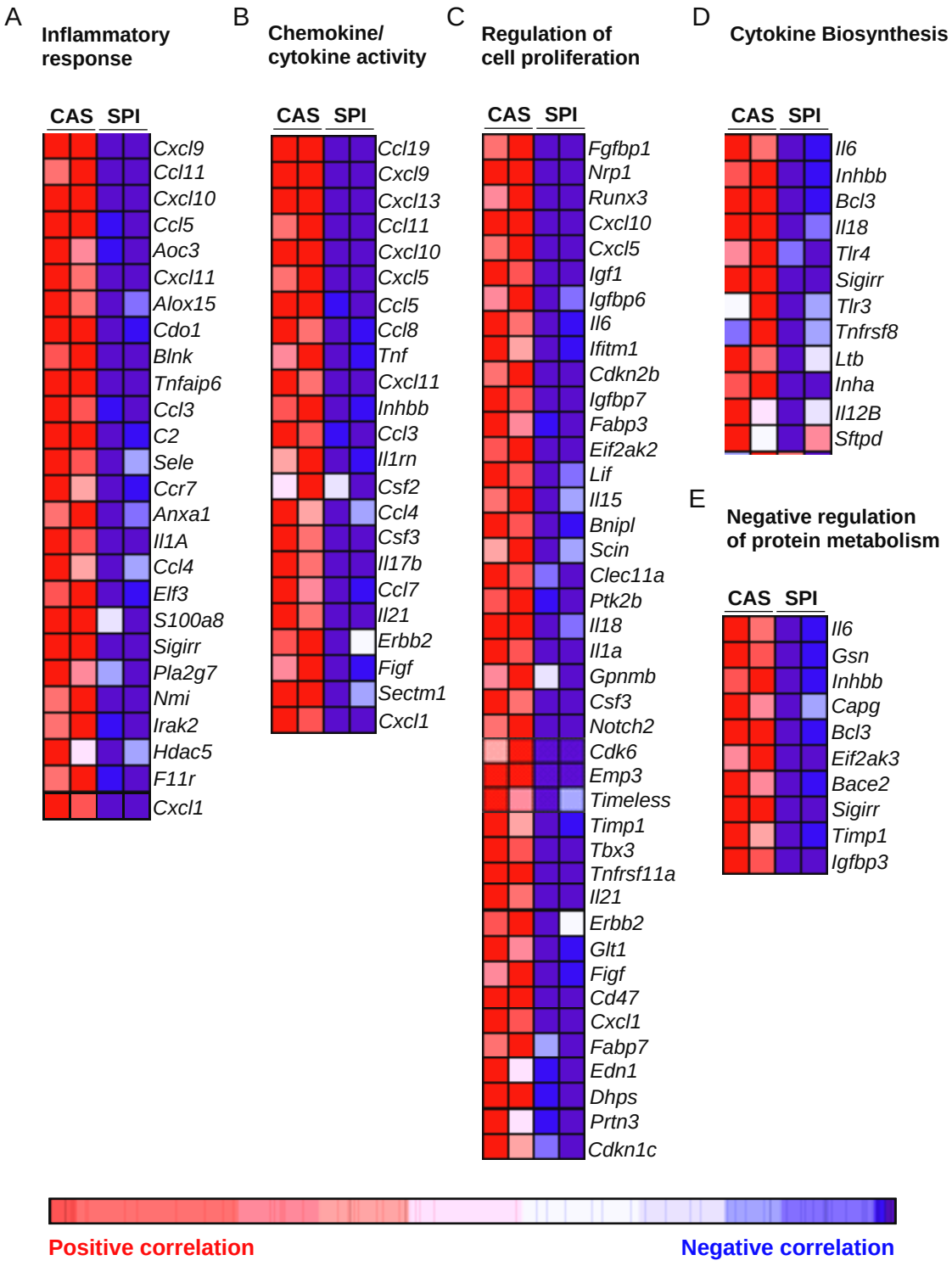

Figure 5. Dietary SPI downregulates MaSC inflammatory, chemokine, and cytokine networks. Heat map representation of SPI-attenuated MaSC transcripts associated with (A) inflammatory response; (B) chemokine/cytokine activity; (C) regulation of cell proliferation; (D) cytokine biosynthesis, and (E) negative regulation of protein metabolism. *Red* indicates positive correlation; *blue*, negative correlation.

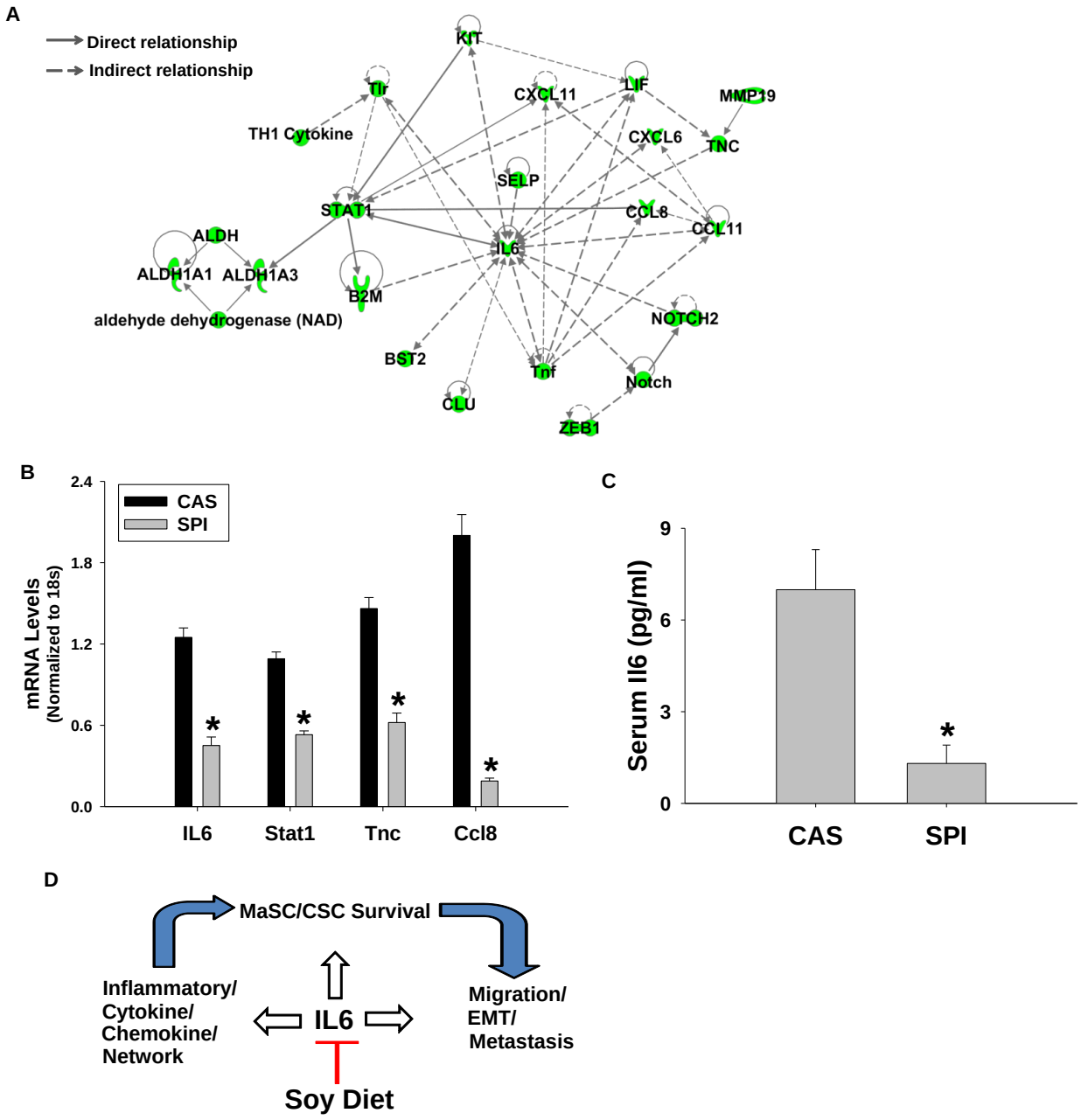

Figure 6. Dietary SPI regulation of molecules related to breast CSC survival, inflammation, and metastasis in MaSC-enriched population of preneoplastic mammary tissues fromWnt1-Tg mice. (A) Molecular network from pathway analysis indicating dietary SPI suppression of molecules involved in MaSC and/or breast CSC survival, inflammatory/chemokine network, and migration/metastasis. *Green* indicates downregulation. (B) QPCR analyses of transcripts for *Il6*, *Stat1* (C), *Ccl8*, and *Tnc* in MaSC-enriched population (n=4-6 independent samples per group) as a function of dietary exposure. *18s* was used as a normalizing control; * $P < 0.05$ relative to CAS. (C) IL6 levels were quantified in sera of PND75 Wnt1-Tg mice fed CAS or SPI; n=13 mice per diet group; * $P < 0.001$ relative to CAS. (D) Proposed model for dietary regulation of MaSC/CSC survival, where IL6 is suggested to be a central target in the inflammatory/metastatic multi-network suppressed by SPI diet.

Manuscript 2: Genistein Inhibits Mammary Tumorigenesis in Wnt1-transgenic Mice by Regulation of Mammary Stem/Progenitor Cells*

* This work has not been submitted to any peer-reviewed journal as of current date.

Dietary GEN inhibits spontaneous mammary tumor formation in Wnt1-Tg mice

We and others have previously shown that dietary protection against chemical-induced mammary tumorigenesis in rats by soy protein isolate (SPI) (2, 4) is recapitulated, in part, by the soy isoflavone GEN (34). Here we used the Wnt1-Tg mice as a model for hereditary breast cancer due to dysregulated Wnt signaling (7) to investigate the effect of dietary GEN on spontaneous mammary tumor formation. While 50% of Wnt1-Tg mice consuming GEN post-weaning developed tumors at 8 months of age, only 22.6% of CAS-fed mice were tumor-free (P= 0.047) (Fig. 7A), without effects on tumor latency (Fig. 7B). Tumor growth within two weeks after detection was not altered by dietary GEN (Fig. 7C) however tumor weights of Wnt1-Tg mice fed GEN were numerically lower (P= 0.14) (Fig. 7D). Wnt1 mRNA levels in mammary tumors did not change with dietary GEN (Fig. 7E). Body weights of Wnt1-Tg mice enrolled in the tumor study were lower in GEN-fed, relative to CAS group (Fig. 7F). Histopathologic analysis of mammary tumors indicated prevalence of solid carcinoma (17) (Table 5).

MaSC-enriched and cancer stem cell populations are decreased by GEN in preneoplastic Wnt1-Tg mice

To investigate whether GEN-mediated protection against mammary tumorigenesis in Wnt1-Tg mice (Fig. 7A) is correlated with regulation of mammary stem/progenitor cells, epithelial subpopulations were quantified in MECs isolated from preneoplastic, virgin PND75 Wnt1-Tg mice fed CAS or GEN by FACS using previously characterized mouse mammary stem cell markers CD29 (β1 integrin) and CD24 (heat stable antigen) within the lineage negative ($CD45^-$, $TER119^-$, $CD31^-$) cells (10). As shown in Figure 8A and 8B, the basal (MaSC-enriched) population ($CD29^{hi}CD24^+$) was decreased (by ~ 2 fold) (P= 0.009) with dietary GEN in preneoplastic PND75 Wnt1-Tg mice, without affecting the luminal ($CD29^{lo}CD24^+$) subset (Fig. 8C). We further quantified Thy1 expression within the basal population to determine possible dietary effects on the previously identified CSCs (Thy1+CD24+) in mammary tumors from Wnt1-Tg mice (11). MECs expressing Thy1 (within basal) were 50% lower in GEN-fed Wnt1-Tg mice, relative to CAS (P= 0.007) (Fig. 8D). These results suggest that decreased percentage of MaSC-enriched population in an oncogenic environment (Wnt1 overexpression) by dietary GEN could inhibit conversion of normal ($CD29^{hi}CD24^+$) to cancer stem cells (Thy1+CD24+) and thus explain mammary tumor protection. Mammary epithelial cell yield was not altered by diet ($2.67 \pm 0.37 \times 10^6$ vs. $2.89 \pm 0.27 \times 10^6$ cells/gram of mammary tissue; CAS vs. GEN, respectively). Transplantation assay developed by DeOme and colleagues is the 'gold standard' to test the self-renewal and thus, regenerative capacity of MaSCs (18). Previous studies showed high *in vivo* repopulating activity of MECs from preneoplastic Wnt1-Tg mice when transplanted into cleared fat pad (10). We transplanted 10,000 MECs isolated from PND75 Wnt1-Tg mice fed either CAS or GEN into three-week old syngeneic WT recipient mice to confirm the presence of MaSCs with *in vivo* regenerative capacity. As shown in Figure 8E, MECs from both CAS- and GEN-fed Wnt1-Tg mice were able to generate a complete mammary epithelial tree upon transplantation, demonstrating presence of MaSCs within the isolated MECs.

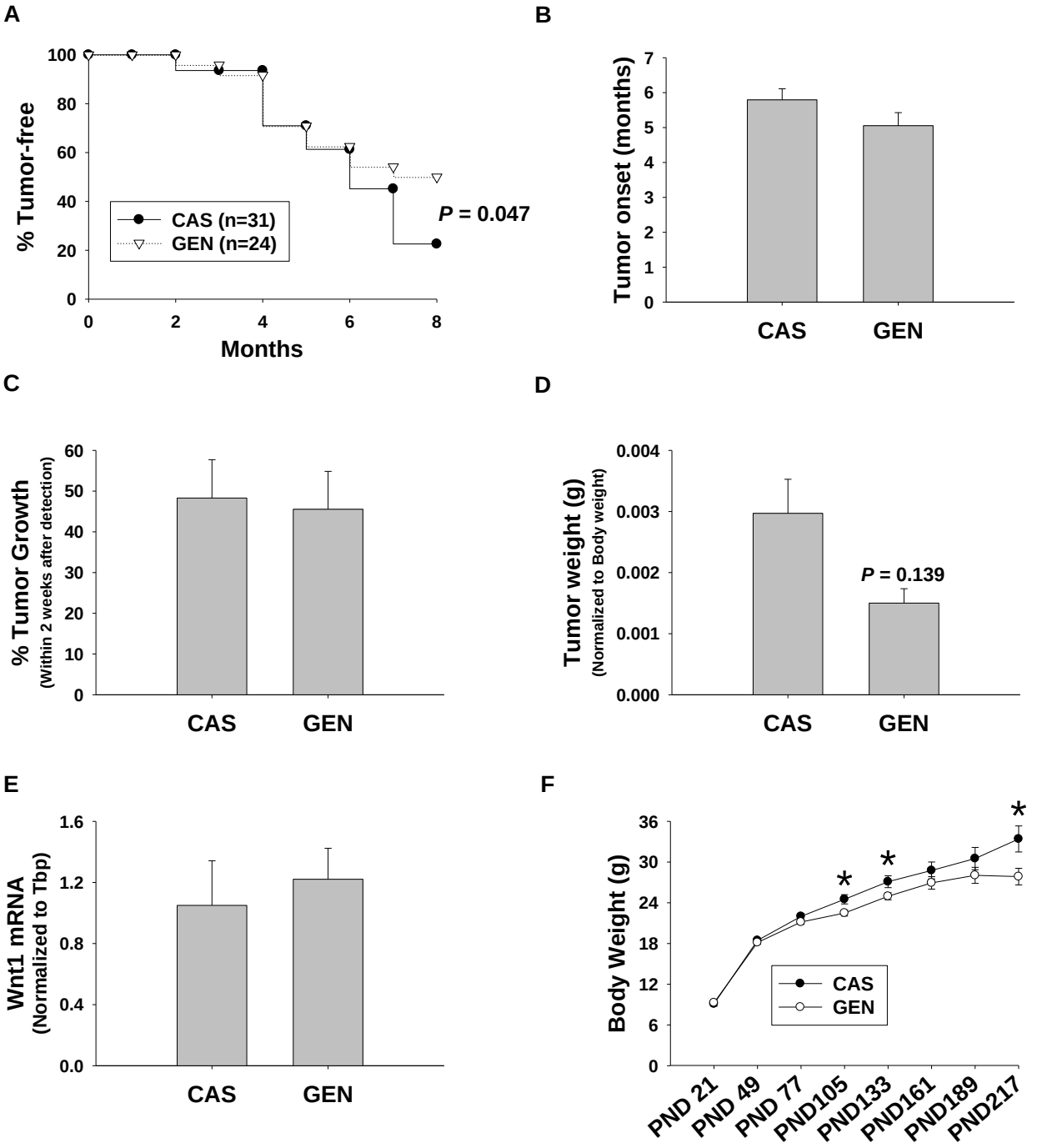

Figure 7. Post-weaning dietary GEN protects against Wnt1-induced mammary tumorigenesis. (A) Percentage of tumor-free Wnt-1Tg female mice fed casein (CAS; n=31) or GEN (n=24). (B) Tumor latency for Wnt1-Tg mice that developed tumors. GEN effects on tumor growth (C) and weight (D). (E) Wnt1 mRNA levels were quantified by QPCR in tumors from Wnt1-Tg mice exposed CAS or SPI diets. *Tbp* was used as a normalizing control. (F) Body weights of Wnt1-Tg mice enrolled in the mammary tumor study were recorded monthly from weaning (PND21) until study conclusion (up to 8 months). * $P < 0.05$ relative to CAS.

Table 5. Histopathologic Features of Mammary Tumors.

Designation	% Incidence	
	CAS[a]	GEN[b]
Solid carcinoma	50.00	42.86
Papillary carcinoma	25.00	21.43
Cribriform carcinoma	5.00	
Adenosquamous carcinoma	5.00	
Acinar carcinoma		7.14
Glandular carcinoma		7.14
Mammary carcinoma in situ	5.00	
Lymph node hyperplasia	5.00	14.29
Lymph node with metastasis	5.00	7.14

[a] n = 20

[b] n = 14

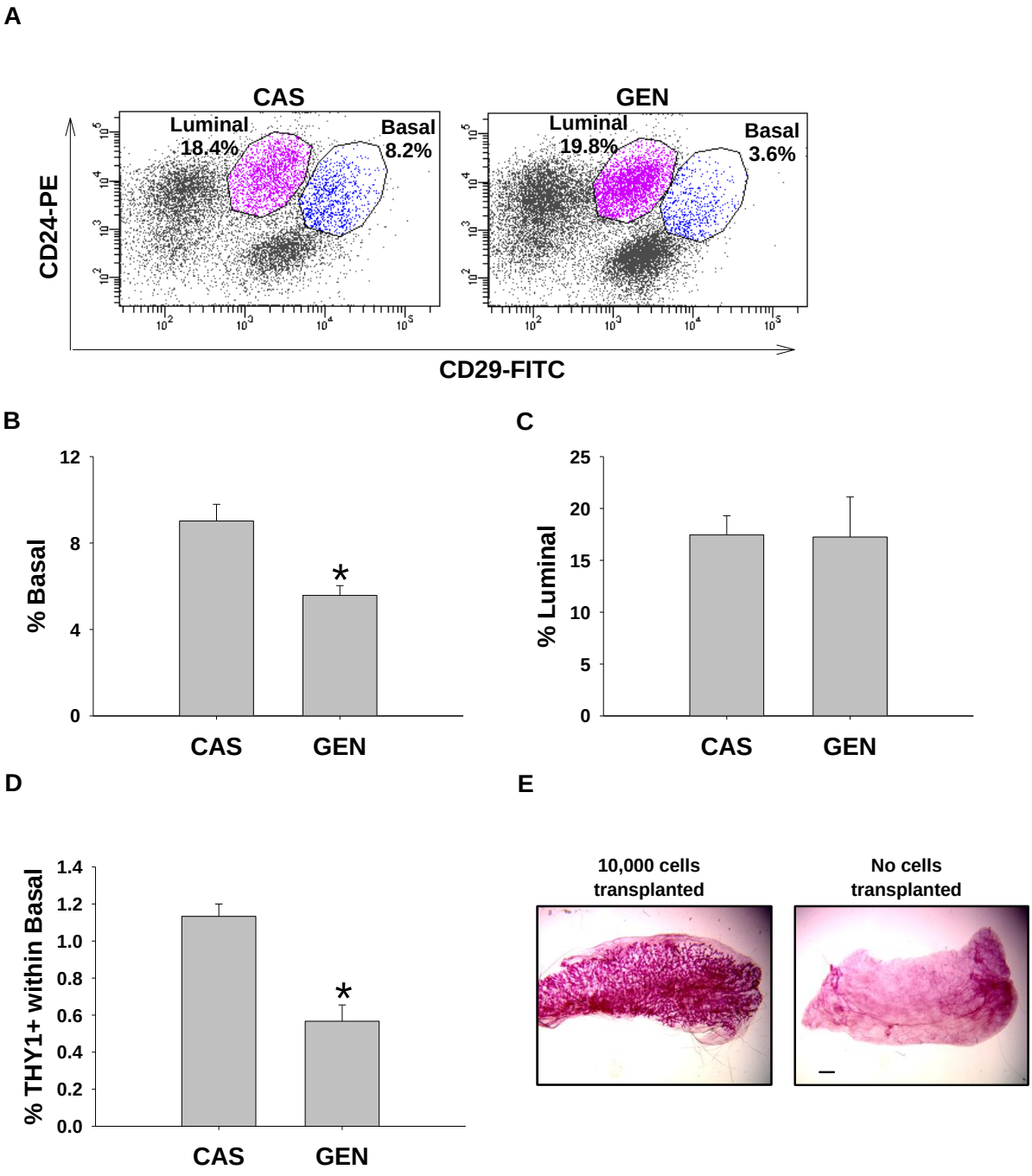

Figure 8. Dietary GEN decreases both basal (MaSC-enriched) and CSC populations in preneoplastic mammary glands of Wnt1-Tg mice. (A) Representative FACS plot of MECs from preneoplastic PND75 Wnt1-Tg mice fed CAS (*Left*) or GEN (*Right*) based on the expression of mouse MaSC markers CD29 and CD24 within the lineage negative ($CD45^-$, $TER119^-$, and $CD31^-$) population after exclusion of dead cells ($DAPI^+$). (B) GEN diet reduced MaSC-enriched ($CD29^{hi}CD24^+$) population without effects on the luminal ($CD29^{lo}CD24^+$) population (C). (D) GEN reduced the cancer stem cell (Thy1+CD24+) population in preneoplastic mammary tissue of Wnt1-Tg mice. Data shown are from at least 5-7 independent experiments. * $P < 0.05$ relative to CAS. (E) *Left*, Whole mounts depicting positive mammary outgrowths from transplantation of freshly isolated MECs (10,000 cells) from preneoplastic mammary glands of Wnt1-Tg mice fed CAS. *Right*, no outgrowths were observed in non-transplanted contralateral mammary gland.

MECs from preneoplastic Wnt1-Tg mice fed GEN have decreased luminal progenitor population

The mouse mammary luminal population ($CD29^{lo}CD24^{+}$) is composed of both luminal progenitors ($CD61^{+}$) and differentiated luminal cells ($CD61^{-}$) (18), where luminal progenitors are enriched for colony-forming activity in Matrigel (21). Further, Vaillant and colleagues (13) demonstrated that luminal progenitors from mammary tumors of Wnt1-Tg mice exhibit tumor-initiating activity. To determine whether mammary tumor protection by GEN (Fig. 7A) is associated with dietary regulation of the mammary luminal progenitors, we measured the ability of MECs to form colonies in Matrigel (Fig. 9A). MECs from pre-neoplastic PND75 Wnt1-Tg mice fed GEN formed lower number of colonies in Matrigel culture (192.14 $\pm$ 8.08 vs. 132.72 $\pm$ 7.00 per 5000 cells; $P<0.001$) (Fig. 9B). These results suggest that the mammary tumor protective effects of GEN also involve regulation of luminal progenitors, in addition to the basal (MaSC-enriched) population.

Dietary GEN decreases mammosphere efficiency of MECs from Wnt1-Tg mice

A mammosphere assay has been developed based on the ability of a small population of mammary stem/progenitor cells to grow in suspension and form spheres while the majority of cells which lack self-renewal ability sink to the bottom of culture plates and die (19). Mammospheres from primary passage (P1) are mainly composed of restricted progenitor cells (20); however, serial passage into secondary mammospheres (P2) enriches for early, bipotent progenitors and stem-like cells (19). We have used the mammosphere assay as a surrogate functional assay to test the effect of diet on the self-renewal of mammary stem/progenitor cells from pre-neoplastic mammary glands of Wnt1-Tg mice fed CAS and GEN diets. As shown in Figure 9C, MECs from GEN-fed Wnt1-Tg mice had lower ability to form both primary and secondary mammospheres, suggesting dual regulation of restricted and early-mammary progenitor/stem cell populations.

Dietary regulation of systemic levels of steroid hormones progesterone and estradiol and IL6

Despite absence of steroid hormone receptors for estrogen (ER) and progesterone (PR) in both human and mouse MaSCs (21, 22), evidence suggest that estrogen and progesterone regulate MaSC activity via paracrine signaling from the luminal compartment (23, 24). To determine whether dietary regulation of MaSC and luminal progenitors are associated with systemic changes in steroid hormones, levels of estradiol-17β and progesterone were measured in sera from PND75 Wnt1-Tg mice used in stem cell analysis and colony formation assay (Figs. 8 and 9). Levels of progesterone were not altered by diet (14.75 $\pm$ 2.11 vs. 15.67 $\pm$ 0.76 ng/ml; Fig. 10A). Interestingly, serum estradiol-17β concentrations were higher in GEN-fed, relative to CAS-fed group (33.07 $\pm$ 1.86 vs. 41.32 $\pm$ 2.71 pg/ml; Fig. 10B).

Serum levels of IL6 levels are consistently higher sera of breast cancer patients, relative to normal counterparts (Knüpfer *et al.* 2007), indicating a negative prognostic marker for serum IL6 in breast cancer. As shown in Figure 10C, serum levels of IL6 were decreased in PND75 Wnt1-Tg mice fed GEN, relative to CAS-fed mice. Results suggest that GEN-mediated regulation of MaSC/CSCs and mammary progenitors may involve in part, downregulation of systemic levels of IL6.

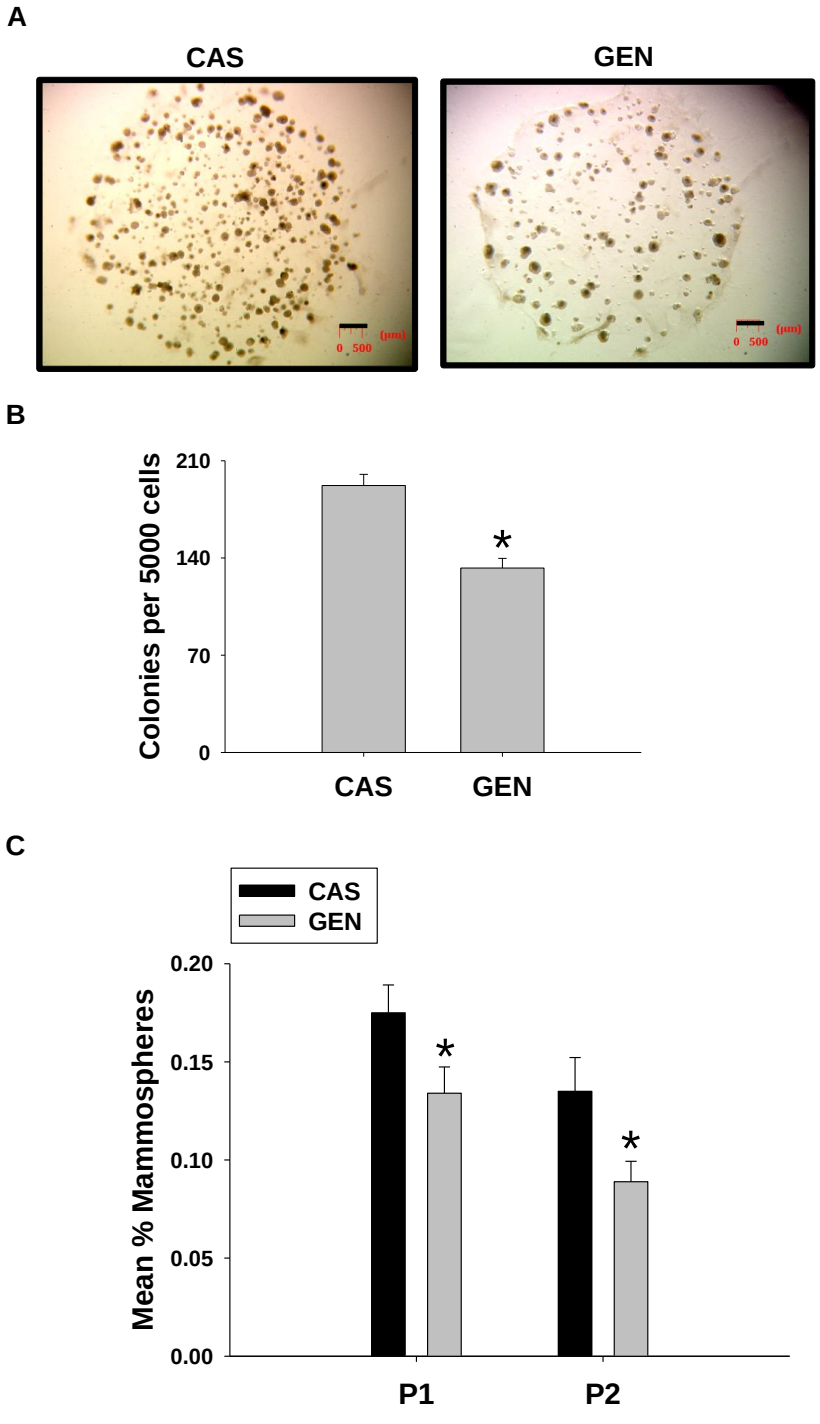

Figure 9. MECs from GEN-fed PND75 Wnt1-Tg mice have decreased colony forming ability and mammosphere forming efficiency. (A) Representative images depicting colony-forming ability of MECs from CAS and GEN-fed PND75 Wnt1-Tg mice. Scale bar, 500 µm. (B) Dietary GEN significantly reduced the colony forming ability of MECs from Wnt1-Tg mice. Histograms show mean ± SEM of five independent experiments (n= 6-12 replicates per experiment/diet). * $P < 0.05$ relative to CAS. (C) MECs from GEN-fed Wnt1-Tg mice have lower ability to form both primary (P1) and secondary (P2) mammospheres. Data represent the mean % mammosphere forming units (MFUs) ± SEM of seven independent experiments (n=16 per experiment); * $P < 0.05$ relative to CAS.

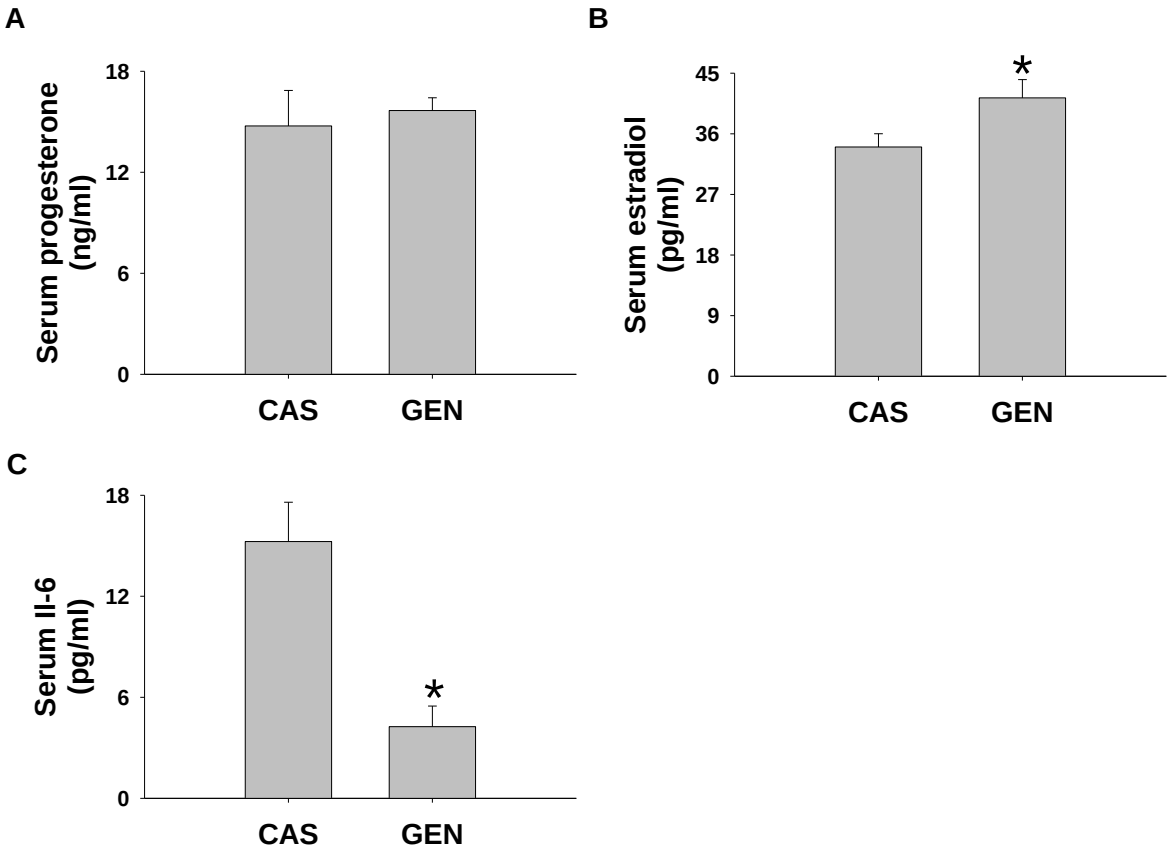

Figure 10. Dietary regulation of systemic levels of steroid hormones and IL6. Serum levels of progesterone (A) and estradiol (B) were measured in PND 75 Wnt1-Tg mice fed CAS or GEN. n=8 and n=15 mice for progesterone and estrogen measurements. (C) IL6 levels were quantified in sera of PND75 Wnt1-Tg mice fed CAS or GEN; n=13 mice per diet group. * $P < 0.05$ relative to CAS.

STATISTICAL METHODS

Data shown for dietary regulation of mouse stem cell markers (CD29 and CD24) by FACS analysis of MECs from pre-malignant (non-tumor) mammary tissue of Wnt-Tg and WT mice represents the average of at least three independent experiments, with MECs pooled from 5-7 mice per experiment, per diet (CAS or SPI). Data is presented as mean $\pm$ standard error of the mean (SEM) for each subpopulation analyzed. Statistical analysis was performed using SigmaStat 3.5 software (SPSS, Chicago, IL). Statistical significance between the treatment diet groups, based on P values ≤ 0.05, was determined using one-way ANOVA followed by Tukey's post hoc analysis. For mammary tumor incidence, up to 30 mice/dietary group were used to allow a significant minimum decrease of ~20% to be detected with 80% power using the one-sided alpha level Chi-square test.

KEY RESEARCH ACCOMPLISHMENTS

- Provided the first direct evidence that PTEN, mainly known to be a phosphatase, can regulate its own expression in MECs by using siRNA, ChIP and promoter assays.
- Identified a novel mechanism for increased differentiation of MECs by soy isoflavone GEN involving a crosstalk between tumor suppressors PTEN and p53.
- Demonstrated that early (prepubertal) lifetime intake of soy foods protects against spontaneous mammary tumor formation in a mouse model of breast cancer relevant to human disease (Wnt-1 overexpressing mice), suggesting a key role for early nutritional regimens in the prevention of adult-onset breast cancer.
- Provided the first *in vivo* evidence, to our knowledge, for dietary regulation of mammary stem cells and its direct association to breast cancer prevention.
- To the best of our knowledge, performed the first gene expression profiling of freshly sorted MaSC-enriched cells from Wnt1-Tg mice as a function of early dietary exposure.
- Demonstrated that protective effects of dietary SPI on spontaneous mammary tumor formation and mammary stem/progenitor cells in Wnt1-Tg mice are mediated in part by soy isoflavone GEN.
- Identified local (MaSC-enriched population) and systemic IL6 production as a central mediator of dietary effects on regulation of mammary stem/progenitor cells and tumor formation. Altered IL6 levels with dietary SPI or GEN intake were correlated with downregulation of a merged network of genes related to CSC survival, inflammation, and metastasis.
- Highlighted diet-regulated, stem cell-associated genes for future potential application in breast cancer therapy.

SIGNIFICANCE

The data generated from this Department of Defense (DOD)-funded study has important implications for individuals with genetic predisposition to breast cancer and could help design better dietary guidelines early in life. Based on extensive evidence supporting a role for cancer stem cells in breast cancer initiation, recurrence, and metastasis, our findings on dietary regulation of stem/progenitor cells in an oncogenic environment such as with overexpression of Wnt-1 oncogene have two main important consequences: 1) they confirm recent human studies demonstrating that consumption of soy-containing whole foods is safe (if not protective) for breast cancer survivors; and 2) they suggest that dietary regulation of mammary stem cell population constitutes an important mechanism contributing to the prevention of adult onset of breast cancer. Thus, distinct dietary regimens/influences early in life may help explain the disparity in breast cancer incidence among the global population.

REPORTABLE OUTCOMES

- Successfully defended my PhD dissertation and completed all the requirements for the Doctor of Philosophy degree in the Interdisciplinary Biomedical Sciences (IBS) Program at the University of Arkansas for Medical Sciences, Little Rock, AR.
- Received and accepted a postdoctoral research position offer at Vanderbilt University Ingram Cancer Center to work towards an academic career with research focus on cancer biology.

- Seven scientific presentations in national meetings (Abstracts listed below) describing studies on the mechanistic linkage between diet and breast cancer risk were supported fully and in part, by the award.
- 5 manuscripts (now published), one Book Chapter (In press), one manuscript in review and one manuscript currently in preparation were supported fully and in part, by the award (All listed below).
- PI was recipient of numerous research and travel awards based on research supported by this fellowship award
    - Third Place–Diet and Cancer Poster Competition at Experimental Biology Meeting, April 22, 2012, San Diego, CA
    - President's Choice-Publication of the Month, October 2011, Arkansas Children's Hospital Research Institute
    - Poster Contest Finalist – Era of Hope Conference, August 2-5, 2011, Orlando Florida
    - Travel Award - University of Arkansas for Medical Sciences Graduate School for attendance at the 2011 Era of Hope Conference (Orlando, Florida)
    - Selected Abstract for Discussion, the 33rd Annual San Antonio Breast Cancer Symposium, December 8-12, 2010, San Antonio, Texas
    - President's Choice-Publication of the Month, September 2010, Arkansas Children's Hospital Research Institute
    - First Place- Senior Division, Interdisciplinary Biomedical Sciences Research Symposium 2010, University of Arkansas for Medical Sciences
    - Achievement Award-University of Arkansas for Medical Sciences, Graduate School (2010)
    - Featured Article (*Research is Key*)-2010 Annual Report of the Arkansas Children's Hospital Research Institute Research

CONCLUSION

Our group has identified the up-regulation of the tumor suppressor PTEN and the inhibition of Wnt-signaling pathway as mechanisms underlying the breast cancer preventive effects of soy foods (2-5). In view of the protective effects of PTEN on Wnt1-induced breast cancer (14, 15) and a role for PTEN/Akt/β-catenin signaling on self-renewal of MaSCs (38) and breast CSCs (11), I examined the exciting hypothesis that dietary regulation of the tumor suppressor PTEN can directly or indirectly alter the progenitor/stem cell population in the mammary gland to confer resistance against breast cancer. My study is highly relevant to our goal of furthering current understanding of the etiology of breast cancer given extensive evidence for the cancer stem cell hypothesis, which suggests that mammary cancer stem cells are not only the origin of breast cancer but also the cause of recurrence and death due to metastasis in affected patients. My studies elucidating the mechanisms for early prevention of breast cancer by diet/dietary factors have great potential to significantly influence public health policies to alleviate the increasing economic, financial, and emotional burdens of breast cancer and other diseases caused by poor nutrition.

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APPENDICES (Publications supported by DoD-BCRP Grant Award)

Abstracts:

1. Rahal O, Pabona JMP, Su Y, Fox SR, Hennings L, Rogers T, Nagarajan S, Simmen RCM. Expansion of Mammary Stem Cell Population with Dietary Intake of Soy Protein Isolate Reveals Novel Mechanisms for Diet-Mediated Control of Mammary Tumorigenesis. 33rd Annual San Antonio Breast Cancer Symposium, December 8-12, 2010. San Antonio, TX. (*Accepted for Poster Discussion*)
2. Montales MT*, Rahal O*, Rogers T, Kang J, Wu X, and Simmen RCM. Repression of Mammosphere Formation in Breast Cancer Cells by Soy Isoflavone Genistein and Blueberry Polyphenols. Experimental Biology, April 9-13, 2011. Washington, DC. (* = equal authorship; *Accepted for Oral Presentation*)
3. Pabona JMP, Dave B, Rahal O, de Lumen BO, de Mejia E, Simmen RCM. Soy Peptide Lunasin Induces PTEN-mediated Apoptosis in Human Breast Cancer Cells. Experimental Biology, April 9-13, 2011. Washington, DC. (*Accepted for Oral Presentation*)
4. Rahal OM, Pabona JMP, Su Y, Fox SR, Hennings L, Rogers T, Nagarajan S, Simmen RCM. Regulation of Mammary Stem Cell Population with Dietary Intake of Soy Protein Isolate Reveals Novel Mechanisms for Diet-Mediated Control of Mammary Tumorigenesis. 2011 Era of Hope conference, August 2-5, 2011. Orlando, FL. (*Poster Contest Finalist*)
5. Simmen RCM, Rahal OM, and Montales MTE. Targeting of Mammary Stem Cells by Dietary Factors in Breast Cancer Prevention. 2011 Era of Hope conference, August 2-5, 2011. Orlando, FL.
6. Rahal OM, Pabona JMP, Hennings L, Prior RL, Kelly T, Al-Dwairi A, Simmen FA, and Simmen RCM. Maternal Blueberry Diet Programs Wnt1-induced Mammary Tumor Progression and Gene Expression in Mouse Offspring. Experimental Biology, April 21-25, 2012. San Diego, CA. (*Accepted for Oral Presentation*)
7. Montales MT, Scanlon S, Matsuda T, Rahal O, and Simmen RCM. Genistein-mediated inhibition of mammary stromal adipocyte differentiation limits expansion of mammary stem/progenitor cells by paracrine signaling. Experimental Biology, April 21-25, 2012. San Diego, CA. (*Accepted for Oral Presentation*)

Manuscripts:

1. Rahal OM, and Simmen RCM. 2010. PTEN and p53 cross-regulation induced by soy isoflavone genistein promotes mammary epithelial cell cycle arrest and lobuloalveolar differentiation. *Carcinogenesis,* 31 (8): 1491-500.
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5. Rahal OM, Pabona JMP, Hennings L, Prior RL, Kelly T, Al-Dwairi A, Simmen FA, and Simmen RCM. 2012. Suppression of Wnt1-induced mammary tumor progression in

offspring by maternal blueberry diet suggests dietary influence on developmental programming. *Carcinogenesis* 34 (2): 464-74.

Book Chapters:

1. Simmen RCM, Rahal OM, Montales MTE, Pabona JMP, Heard ME, Al-Dwairi A, Brown AR, Simmen FA. 2013. Soy Foods: Towards theDevelopment of Novel Therapeutics for Breast Cancer. In: Cancer Chemoprevention and Treatment by Diet Therapy. W Cho (editor), Springer Press (In press).

APPENDIX 1: First author publication in *Carcinogenesis* journal (2010) (added at the end of the report).

APPENDIX 2: Abstract accepted for *Poster Discussion* at the 33rd Annual San Antonio Breast Cancer Symposium, December 8−12, 2010.

Regulation of Mammary Stem Cell Population with Dietary Intake of Soy Protein Isolate Reveals Novel Mechanisms for Diet-Mediated Control of Mammary Tumorigenesis.

Rahal O, Pabona JMP, Su Y, Fox SR, Hennings L, Rogers T, Nagarajan S, Simmen RCM. Arkansas Children's Nutrition Center and University of Arkansas for Medical Sciences, Little Rock, AR

Breast cancer risk is highly modified by environmental factors including diet. Previously, we showed that dietary intake of soy protein isolate (SPI) decreased mammary tumor incidence and increased mammary tumor latency in rats relative to those fed a control casein (CAS) diet, when exposed to the chemical carcinogen NMU. Mammary tumor preventive effects by SPI were associated with up-regulation of the tumor suppressor PTEN and down-regulation of the oncogenic Wnt-signaling components in mammary epithelial cells (MECs) leading to enhanced differentiation. Given that breast cancer is considered to be initiated by stem cells (SCs) with tumorigenic potential, termed cancer stem cells (CSCs), and mammary over-expression of Wnt-1 in mice causes spontaneous breast tumors due to the expansion of mammary CSCs, we hypothesized that diet may alter the mammary SC population to effect mammary tumor prevention. Here, we investigated SPI effects relative to CAS, on mammary tumor development in MMTV-Wnt 1-Transgenic (Tg) female mice and on the mammary SC population in virgin wildtype (WT) and pre-neoplastic Tg female mice. Tumor incidence at 8 months of age of Tg mice fed SPI (n=30) post-weaning was lower than in those fed CAS (48.3% vs.73.5%; $p<0.05$) (n=34). Interestingly, tumor latency in SPI-fed Tg mice was shorter than for the CAS-fed group (4.65 vs. 5.88 months; $P<0.05$). Tumor weight and growth rate was similar for the diet groups. To evaluate SPI effects relative to CAS, on mammary SC population, epithelial cells from mammary tissues were isolated from WT (PND 100) and Tg (PND75) mice. The percentage of mammary SCs was quantified by fluorescence activated cell sorting analysis of MECs based on their expression of mouse mammary SC markers (CD29 and CD24) within the Lineage negative (Lin^-) population ($CD45^-$, $TER119^-$, $CD31^-$). The $Lin^-CD29^{hi}CD24^{hi}$ subpopulation in MECs was decreased by 50% in Tg mice fed SPI post-weaning relative to those fed CAS, decreasing the likelihood of mutations that convert normal to cancer SC and could explain the protective effects of SPI on tumor incidence. Interestingly, the SC population was expanded by 2-fold in MECs of WT mice fed SPI relative to the CAS group, which could be beneficial for mammary gland development and tissue homeostasis. Our findings provide the first report of dietary effects on the SC population in MECs *in vivo.* The dichotomy of SPI effects on tumor outcome in mammary tissues with dysregulated Wnt signaling maybe related to the loss of the complex regulatory grid between PTEN and Wnt/β-catenin pathways, both of which control stem cell fate. The possibility that diet can influence tumor progression at the level of the SC population suggests the important contribution of nutrition to the etiology of breast cancer and to the early management of breast health. Supported by USDA- ARS and Department of Defense Breast Cancer Research Program.

APPENDIX 3: Co-authored abstract presented at the 2011 Experimental Biology Meeting. April 9-13, 2011. Washington, DC (*Accepted for Oral Presentation*)

Repression of Mammosphere Formation in Breast Cancer Cells by Soy Isoflavone Genistein and Blueberry Polyphenols

Maria Theresa Montales[1,2], Omar Rahal[1,3], Theodore Rogers[1], Jie Kang[1], Xianli Wu[1] and Rosalia CM Simmen[1,2]. [1]Arkansas Children's Nutrition Center, [2]Physiology & Biophysics and [3]Interdisciplinary Biomedical Sciences, University of Arkansas for Medical Sciences, Little Rock, AR.

Epidemiological evidence implicates diets rich in fruits and vegetables in breast cancer prevention due to their phytochemical components, yet mechanisms underlying their presumed anti-tumor activities are not well-understood. A small population of mammary epithelial cells, termed cancer stem cells (CSC), may be responsible for initiating and sustaining tumor development. To evaluate dietary components that selectively target CSC and thus, provide mammary tumor protection, we utilized the estrogen receptor-positive MCF-7 and estrogen receptor-negative MDA-MB231 human breast cancer cell lines. Within 5 days of culture, both cell lines formed mammospheres at a frequency (1-2%) consistent with a subset of the cell population exhibiting stem cell-like characteristics. The soy isoflavone genistein dose-dependently decreased (40 nM > 2 μM; by 2-3-fold) mammosphere numbers from both cell lines, relative to medium alone. A mixture of phenolic acids that include hippuric acid, ferrulic acid and 3-hydroxycinnamic acid, based on concentrations found in sera of rats fed diets containing 10% blueberry similarly inhibited (by 2-fold) mammosphere formation in MDA-MB231 but not in MCF-7 cells. The adipokine leptin and the inflammatory cytokine interleukin-6 had no activity in these cells. Findings suggest that dietary factors may target cancer cells with stem-like properties in the prevention of breast cancer.
Grant Funding Source: USDA-CRIS 6251-51000-005-02S; Department of Defense Breast Cancer Research Program 0810548 (RCMS) and W81XWH-10-1-0047 (OR)

APPENDIX 4: Co-authored abstract presented at the 2011 Experimental Biology Meeting. April 9-13, 2011. Washington, DC (*Accepted for Oral Presentation*)

Soy peptide lunasin induces PTEN-mediated apoptosis in human breast cancer cells

John Mark Pascua Pabona[1,2], Bhuvanesh Dave[3], Omar Rahal[1,2], Ben O de Lumen[4], Elvira de Mejia[5], Rosalia CM Simmen[1,2]. [1]Arkansas Children's Nutrition Center, Little Rock, AR, [2]University of Arkansas for Medical Sciences, Little Rock, AR, [3]The Methodist Hospital Research Institute, Houston, TX, [4]University of California, Berkeley, CA, [5]University of Illinois, Urbana-Champaign, IL

The tumor suppressor PTEN inhibits the AKT signaling pathway whose unrestrained activity underlies many human malignancies. Previously we showed that dietary intake of soy protein isolate (SPI) enhanced PTEN expression in mammary tissue of rats with lower NMU-induced mammary tumor incidence relative to those fed casein-based diet. While epidemiological studies corroborate the breast cancer protective effects of soy, specifically of the major soy isoflavone genistein (GEN), the identity of other bioactive soy components remains relatively unknown. Here we evaluated the effects of lunasin, a soybean peptide previously detected in sera of rats and humans consuming soy-rich diets, on PTEN-mediated apoptosis of the mammary carcinoma cell line MCF-7. Lunasin (2μM >50 nM) increased PTEN expression and nuclear localization (by 2.5-fold); enhanced PTEN-mediated cellular apoptosis (by 10-15-fold); and altered levels of p53 (increased) and p21WAF1 (decreased) transcripts ($P<0.05$). GEN (2 μM >20 nM) elicited similar effects as lunasin on PTEN expression and PTEN-mediated apoptosis in MCF-7 cells. Lunasin and GEN are known to regulate core histone acetylation by which PTEN promoter activity is similarly controlled. Findings suggest that activation of PTEN expression by bioactive soy components, possibly via epigenetic mechanisms may underlie breast cancer protection. [USDA-CRIS; Department of Defense BCRP]

APPENDIX 5: Co-authored abstract submitted to the 2011 Era of Hope Conference. August 2-5, 2011. Orlando, FL.

Targeting of Mammary Stem Cells by Dietary Factors in Breast Cancer Prevention

Simmen RCM, Rahal O., and Montales, M.T, Arkansas Children's Nutrition Center and University of Arkansas for Medical Sciences, Little Rock, AR 72202.

Breast cancer is the most common malignancy of women in the Western world, with ~50,000 of those afflicted dying annually from the disease. Although many risk factors are associated with the development and progression of breast cancer, diet/nutrition constitutes a highly modifiable risk. Breast cancer is increasingly acknowledged to be initiated by mutations in a limited population of undifferentiated cells termed stem cells that 'sit' at the top of the mammary epithelial hierarchy. Over-expansion of the stem cell population can alter the balance of cell proliferation and differentiation and thus, increase the number of mutated cells that can initiate and maintain tumors which eventually metastasize. Novel strategies to decrease the over-expansion and promote the elimination of tumor-initiating cells are warranted for the effective treatment and prevention of breast cancer. Our studies test the hypothesis that dietary factors confer protection from breast cancer by preventing the expansion of stem/progenitor cells with tumorigenic potential. We established female mice transgenic for the oncogene Wnt-1 (Wnt-Tg), which develop spontaneous mammary tumors by 6-8 months of age, as a model system for dietary prevention of mammary tumor formation. Mice were fed American Institute of Nutrition-based isocaloric diets that differed only by protein source, namely Casein (CAS) and soy protein isolate (SPI). SPI was used as a paradigm for healthy foods, given the epidemiological linkage of decreased breast cancer incidence in women with high consumption of soy-rich foods. Lifetime dietary exposure to SPI beginning at pre-puberty resulted in lower mammary tumor incidence ($P<0.05$) at 8 months of age in Wnt-Tg females (48.3%; n=30), relative to those fed the control CAS diet (73.5%; n=34). Analyses of the mammary epithelial stem cell population by flow cytometry, using established mouse mammary stem cell markers CD29 and CD24 ($CD29^{hi}CD24^{+}$) within the Lineage-negative population indicated decreased number of stem cells (by 50%; $P<0.05$) in mammary glands of Wnt-Tg mice fed SPI. The mammosphere-forming efficiency of mammary epithelial cells isolated from mammary glands of SPI-fed mice was similarly lower (by 2-fold; $P<0.05$) than in those of mice fed CAS. To identify dietary component(s) that target stem cells to inhibit mammary tumor formation, we evaluated the ability of the major soy isoflavone genistein (GEN), a phytoestrogen, to inhibit mammosphere formation in two human breast cancer cell lines, namely the estrogen receptor (ER)-positive MCF-7 and ER-negative MDA-MB231. Cells plated in ultralow attachment plates formed mammospheres at a frequency of 1-2% within 5 days of seeding. GEN at physiological doses (40 nM>2 μM) decreased the number of mammosphere-forming units in both cell lines, relative to medium alone. Our studies demonstrate a functional (inverse) connection between exposure to a 'healthy' diet during early life and abundance of mammary stem cells, providing strong support for healthy dietary strategies in young children to decrease incidence of breast cancer during adulthood. Further, our work established the formation of mammospheres as a promising diagnostic tool for evaluating dietary factors with mammary tumor-inhibiting potential, which may help improve the efficacy of traditional chemotherapy. **Funding Support**: DoD-BCRP-W81XWH-08-0548 (RCMS), USDA-CRIS 6251-5100002-06S (RCMS), and DoD-BCRP Predoctoral fellowship W81XWH-10-1-0047 (OR).

APPENDIX 6: Abstract submitted to the 2011 Era of Hope Conference. August 2-5, 2011. Orlando, FL. (*Poster Contest Finalist)*

BC093685-2620

REGULATION OF MAMMARY STEM CELL POPULATION WITH DIETARY INTAKE OF SOY PROTEIN ISOLATE REVEALS NOVEL MECHANISMS FOR DIET-MEDIATED CONTROL OF MAMMARY TUMORIGENESIS

Omar Rahal
Arkansas Children's Hospital Research Institute

Breast cancer risk is highly modified by environmental factors including diet. Previously, we showed that dietary intake of soy protein isolate (SPI) decreased mammary tumor incidence and increased mammary tumor latency in rats relative to those fed a control casein (CAS) diet when exposed to the chemical carcinogen NMU. Mammary tumor preventive effects by SPI were associated with upregulation of the tumor suppressor PTEN and downregulation of the oncogenic Wnt-signaling components in mammary epithelial cells (MECs) leading to enhanced differentiation. Given that breast cancer is considered to be initiated by stem cells (SCs) with tumorigenic potential, termed cancer stem cells (CSCs), and mammary overexpression of Wnt-1 in mice causes spontaneous breast tumors due to the expansion of mammary CSCs, we hypothesized that diet may alter the mammary SC population to effect mammary tumor prevention. Here, we investigated SPI effects relative to CAS, on mammary tumor development in MMTV-Wnt1-Transgenic (Wnt1-Tg) female mice and on the mammary SC population in virgin wild-type (WT) and pre-neoplastic Wnt1-Tg female mice. Tumor incidence at 8 months of age of Wnt1-Tg mice fed SPI (n=30) post weaning was lower than in those fed CAS (48.3% vs.73.5%; $p<0.05$) (n=34). Interestingly, tumor latency in SPI-fed Wnt1-Tg mice was shorter than for the CAS-fed group (4.65 vs. 5.88 months; $P<0.05$). Tumor weight and growth rate were similar for the diet groups. To evaluate SPI effects relative to CAS, on mammary SC population, epithelial cells from mammary tissues were isolated from Wnt1-Tg (PND75) mice. The percentage of mammary SCs was quantified by fluorescence-activated cell sorting analysis of MECs based on their expression of mouse mammary SC markers (CD29 and CD24) within the lineage negative (Lin-) population (CD45-, TER119-, CD31-).The Lin-CD29hiCD24hi subpopulation in MECs was decreased by 50% in Wnt1-Tg mice fed SPI post weaning relative to those fed CAS, decreasing the likelihood of mutations that convert normal to CSCs and could explain the protective effects of SPI on tumor incidence. Interestingly, the SC population was expanded by 2-fold in MECs of WT mice fed SPI relative to the CAS group, which could be beneficial for mammary gland development and tissue homeostasis. Our findings provide the first report of dietary effects on the SC population in MECs in vivo. The dichotomy of SPI effects on tumor outcome in mammary tissues with dysregulated Wnt signaling may be related to the loss of the complex regulatory grid between PTEN and Wnt/b-catenin pathways, both of which control SC fate. The possibility that diet can influence tumor progression at the level of the SC population suggests the important contribution of nutrition to the etiology of breast cancer and to the early management of breast health. *This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-10-1-0047, the U.S. Department of Agriculture, and Agricultural Research Service.*

APPENDIX 7: Co-author publication (review article) in *Journal of Nutritional Biochemistry* (2011) (added at the end of the report).

APPENDIX 8: First author publication in *Endocrinology* journal (2011) (added at the end of the report).

APPENDIX 9: Co-first author* publication in *Carcinogenesis* journal (2012) (added at the end of the report). * = equal contribution.

APPENDIX 10: Co-author in abstract accepted for *Oral Presentation* and *Poster Competition* (Second Place) at the Experimental Biology Meeting, April 22, 2012, San Diego, CA.

Genistein-mediated inhibition of mammary stromal adipocyte differentiation limits expansion of mammary stem/progenitor cells by paracrine signaling

Maria Theresa Montales, Samantha Scanlon, Tsukasa Matsuda, Omar Rahal, and Rosalia C.M. Simmen. Arkansas Children's Nutrition Center and University of Arkansas for Medical Sciences, Little Rock, AR and Nagoya University, Japan.

Mammary adiposity may contribute to breast cancer development and progression by the release of cytokines and other inflammatory mediators that promote mammary epithelial proliferation and inhibit differentiation. We evaluated the effects of genistein (GEN), a major component of soy foods with breast cancer protective actions, on the adipogenic differentiation of a SV40-immortalized mouse mammary stromal fibroblast like cell line (MSF) treated with differentiating agents insulin, hydrocortisone and troglitazone (DM). MSF cultured in DM with and without extracellular matrix for 14 days showed reduced differentiation into mature adipocytes (2-3 fold) with 40nM but not 2uM GEN, as evaluated by Oil Red O staining. Addition of sera from GEN-fed adult mice to MSF similarly reduced adipogenic differentiation (4-5 fold), relative to sera from casein-fed mice. Lipid accumulation was decreased by GEN (40nM>2uM). Expression levels of lipogenic enzyme genes fatty acid synthase and peroxisome proliferator-activated receptor-γ were down-regulated by 40nM GEN. By contrast, 40 nM GEN increased estrogen receptor (ER)-β but not ER-α expression. Conditioned media from GEN-treated MSF reduced mammosphere formation of human breast cancer MCF-7 cells. Results suggest that dietary factor control of mammary adiposity may influence mammary tumor development for breast cancer prevention. Funding: USDA-CRIS, DOD, and CUMG/ACHRI.

APPENDIX 11: Abstract accepted for *Oral Presentation* and *Poster Competition* (Third Place) at the Experimental Biology Meeting, April 22, 2012, San Diego, CA.

Maternal blueberry diet suppresses Wnt1-induced mammary tumor progression in offspring

Omar M. Rahal, John Mark Pabona, Leah Hennings, Ronald L. Prior, Thomas Kelly, Ahmed Al-Dwairi, Frank A. Simmen, and Rosalia C.M. Simmen. Arkansas Children's Nutrition Center and University of Arkansas for Medical Sciences

Despite the well-accepted notion of peri-natal origins of adult diseases, the factors and regulatory mechanisms underlying breast cancer development remain unclear. Diet is a highly modifiable determinant of breast cancer risk, and the effects of the *in utero* nutritional environment persist beyond fetal life. We investigated whether *in utero*/lactational exposure to blueberry (BB) via maternal diet alters the trajectory of Wnt1-induced mammary tumorigenesis in offspring. Wnt1 transgenic mice were exposed to maternal diets of casein (CAS; n=33) or blueberry-supplemented CAS (3% BB; n=28) from gestation day 4 until post-natal day 21. Offspring were then weaned to CAS and mammary tumor development was followed until age 8 months. While tumor incidence and latency were similar for both groups, tumor weight (by 2-fold, p=0.034) and growth rate (by 60%; p=0.008) were reduced in offspring of BB- versus CAS-fed dams. Tumors from the BB group had higher expression of tumor suppressors PTEN and E-cadherin and lower cyclin D1 and pro-apoptotic Bcl2 levels. Transcript levels for DNA methylation enzymes DNMT1 and EZH2 were higher in BB tumors. Serum levels of insulin and of leptin/adiponectin ratio were lower for tumor-bearing BB than CAS offspring at sac. Our findings support a role for nutritional epigenetics in adult breast cancer outcome.

Funding: USDA-CRIS (RCMS), DOD-BCRP (OMR) and NIH-NCI (FAS).

APPENDIX 12: First author publication in *Carcinogenesis* journal (2013) (added at the end of the report).

Carcinogenesis vol.31 no.8 pp.1491–1500, 2010
doi:10.1093/carcin/bgq123
Advance Access publication June 16, 2010

PTEN and p53 cross-regulation induced by soy isoflavone genistein promotes mammary epithelial cell cycle arrest and lobuloalveolar differentiation

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The tumor suppressors phosphatase and tensin homologue deleted on chromosome ten (PTEN) and p53 are closely related to the pathogenesis of breast cancer, yet pathway-specific mechanisms underlying their participation in mediating the protective actions of dietary bioactive components on breast cancer risk are poorly understood. We recently showed that dietary exposure to the soy isoflavone genistein (GEN) induced PTEN expression in mammary epithelial cells *in vivo* and *in vitro*, consistent with the breast cancer preventive effects of soy food consumption. Here, we evaluated PTEN and p53 functional interactions in the nuclear compartment of mammary epithelial cells as a mechanism for mammary tumor protection by GEN. Using the non-tumorigenic human mammary epithelial cells MCF10-A, we demonstrate that GEN increased PTEN expression and nuclear localization. We show that increased nuclear PTEN levels initiated an autoregulatory loop involving PTEN-dependent increases in p53 nuclear localization, PTEN–p53 physical association, PTEN–p53 co-recruitment to the *PTEN* promoter region and p53 transactivation of *PTEN* promoter activity. The PTEN–p53 cross talk induced by GEN resulted in increased cell cycle arrest; decreased pro-proliferative *cyclin D1* and *pleiotrophin* gene expression and the early formation of mammary acini, indicative of GEN promotion of lobuloalveolar differentiation. Our findings provide support to GEN-induced PTEN as both a target and regulator of p53 action and offer a mechanistic basis for PTEN pathway activation to underlie the antitumor properties of dietary factors, with important implications for reducing breast cancer risk.

Introduction

Breast cancer is the most common malignancy among women in the Western world, affecting one of eight in their lifetime and resulting in ~50 000 deaths in the USA annually (1). Accumulations of epigenetic and genetic alterations within mammary epithelial cells (MECs) are the triggering events for breast cancer initiation and tumor cell expansion (2). Prevailing evidence suggests that breast cancer development can be influenced by nutrition (3). Epidemiological and case–control studies have shown a 2- to 8-fold lower occurrence of the disease in Asian women whose early intake of soy products is 10–20 times higher than their American counterparts (4,5). Several human, animal and *in vitro* studies concur that early exposure (prepubertal) to soy foods and associated components is correlated with reduced risk of adult breast cancer (6,7). Among the soy products, the isoflavone genistein (GEN) has been identified as an important component that may confer protection against breast tumors (8–10).

Abbreviations: CAS, casein; EGF, epidermal growth factor; ER, estrogen receptor; GEN, genistein; MEC, mammary epithelial cells; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homologue deleted on chromosome ten; PCR, polymerase chain reaction; QPCR, quantitative real-time polymerase chain reaction; scRNA, scrambled RNA; siRNA, small interfering RNA; SPI, soy protein isolate.

Previously, we (11,12) and others (9,13) showed that dietary intake of soy protein isolate (SPI) and control casein (CAS) supplemented with GEN decreased mammary tumor incidence and/or increased tumor latency in rats fed these diets relative to those fed CAS, when exposed to the chemical carcinogens N-methyl-N-nitrosourea or 7,12-dimethyl-benz[*a*]anthracene. In our studies, breast cancer protective effects were associated with the downregulation of the oncogenic Wnt-signaling pathway and the upregulation of the phosphatase and tensin homologue deleted on chromosome ten (*PTEN*) expression in the mammary gland by SPI and GEN, relative to CAS, coincident with enhanced MEC differentiation (14,15). Given that these same diets altered several biological and molecular pathways in MECs *in vivo* (14), additional pathways probably underlie their mammary tumor protective effects.

Next to p53, PTEN is the most common tumor suppressor to be lost or inactivated in human cancers, including breast cancer (16,17). The *PTEN* gene encodes a dual specificity (lipid and protein) phosphatase that antagonizes phosphatidylinositol 3-kinase (PI3K), preventing activation of the pro-survival protein kinase B/Akt downstream pathway (18). A role for PTEN in mammary gland development and tumorigenesis is supported by reduced cellular proliferation and increased apoptosis in the mammary glands of mice overexpressing PTEN (19). Further, Cowden syndrome patients harboring germ line mutations at the *PTEN* locus are at high risk of breast cancer and although somatic mutations of *PTEN* are found in a small fraction of breast cancers (20,21), loss of heterozygosity at the *PTEN* locus (10q23) occurs frequently (22). Additional studies linking breast carcinoma status with PTEN expression include those demonstrating the predictive value of reduced PTEN in the relapse of tamoxifen-treated estrogen receptor (ER)-α-positive breast cancer patients (23) and the prognostic significance of the gene expression signature of PI3K/Akt activation due to PTEN loss on metastasis and poor survival (24).

One mechanism by which PTEN may protect against mammary tumors is by its interaction with the tumor suppressor p53. PTEN transcription can be enhanced by p53 (25), in turn, PTEN regulates p53 protein stability in two ways: in a phosphatase-dependent manner, by inhibiting PI3K/Akt-induction of Mdm2 nuclear translocation and in a phosphatase-independent manner through its physical interaction with p53 (26). Perhaps, the most convincing evidence supporting complementary functions of PTEN and p53 in tumorigenesis is the unexpected observation that PTEN is oncogenic in the presence of a mutant p53 protein (27).

In this study, we explored a novel mechanism of breast cancer prevention by GEN involving PTEN and p53. Using the non-malignant human mammary epithelial cell line MCF-10A, we show that GEN at physiologically relevant concentrations induced PTEN expression and PTEN and p53 nuclear accumulation. We demonstrate that GEN increased nuclear PTEN expression through an autoregulatory loop whereby PTEN's increased interaction with nuclear p53 enhanced *PTEN* promoter activity. Further, we establish that a functional consequence of augmented nuclear PTEN signaling by GEN was the promotion of cell cycle arrest and the stimulation of early lobuloalveolar differentiation. Our results point to PTEN as both a target and regulator of p53 action in normal MECs for mammary tumor prevention by the isoflavone GEN.

Materials and methods

Animals, diets and MEC isolation

Animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences. Time-mated Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) were individually housed in polycarbonate cages under conditions of 24°C, 40%

humidity and a 12 h light–dark cycle. At gestation day 4, dams were randomly assigned to one of two semi-purified isocaloric diets made according to the American Institute of Nutrition-93 G formulation (28), with corn oil substituting for soybean oil. These diets are: (i) CAS diet, containing casein (New Zealand Milk Products, Santa Rosa, CA) as the only protein source and (ii) SPI diet containing soy protein isolate (Solae, St Louis, MO) with isoflavones GEN (216 ± 2 mg/kg) and daidzein (160 ± 6 mg/kg) as aglycone equivalents. Female pups were weaned to the same diets as their dams until isolation of MECs at postnatal day 50. Mammary gland pair #3 was processed for MEC isolation following protocols described by Dr Jeffrey Rosen's laboratory (http://www.bcm.edu/rosenlab/protocols/primaryMEC.pdf; Baylor College of Medicine, Houston, TX).

Cell culture and treatments

The human non-tumorigenic mammary epithelial cell line, MCF-10A (American Type Culture Collection, Manassas, VA), was propagated as described (29). Phenol red-free media supplemented with charcoal-stripped fetal bovine serum was used for serum starvation (0.5% charcoal-stripped fetal bovine serum) and for treatments (2.5% charcoal-stripped fetal bovine serum) with GEN (Sigma Chemical Co., St Louis, MO) or vehicle (dimethyl sulfoxide). For small interfacing RNA (siRNA) targeting studies, cells were grown to 30–50% confluency prior to transfecting with PTEN siGENOME SMART pool or siCONTROL Non-targeting siRNA pool (Dharmacon, Lafayette, CO) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), following the manufacturer's protocol.

Quantitative real-time polymerase chain reaction

Total RNA was isolated from cells using Trizol reagent (Invitrogen), quantified and reverse transcribed to complementary DNA as described (14). SYBR Green detection system (Applied Biosystems, Foster City, CA) was used for quantitative real-time polymerase chain reaction (QPCR). Human primer sequences and corresponding genes were [gene, forward and reverse primer; amplicon size (bp)]: *PTEN* (5′-GCTATGGGATTTCCTGCAGAA-3′ and 5′-GGCGGTGTCATAATGTCTTTCA-3′; 138), *p53* (5′-GGCGCACAGAGGAAGAGAAT-3′ and 5′-GGAGAGGAGCTGGTGTTGTTG-3′; 103), *18S* (5′-TCTTAGCTGAGTGTCCCGCG-3′ and 5′-ATCATGGCCTCAGTTCCGA-3′; 151), *pleiotrophin* (5′-TGCCAGAAGACTGTCACCATCT-3′ and 5′-TCCTGTTTCTTGCCTTCCTTTT-3′; 101) and *cyclin D1* (5′-AATGACCCCGCACGATTTC-3′ and 5′-ATGGAGGGCGGATTGGAA-3′; 144). Rat primers used were: *PTEN* (5′-CAATGTTCAGTGGCGGAACTT-3′ and 5′-GGCAATGGCTGAGGGAACT-3′; 133) and *18S* (5′-ATTCGAACGTCTGCCCTATCAA-3′ and 5′-CGGGAGTGGGTAATTTGCG-3′; 151). 18S ribosomal RNA was used as a normalizing control and data are expressed as means ± SEM relative to control (vehicle).

Immunoprecipitation and immunoblotting

Immunoprecipitation was performed in whole cell lysates using the Catch and Release Immunoprecipitation System following the manufacturer's instructions (Upstate Biotechnology, Lake Placid, NY). Briefly, 500 μg of cell lysate were incubated with 2 μg of PTEN antibody (A2B1; Santa Cruz, Santa Cruz Biotechnology, CA), p53 antibody (Cell Signaling Technology, Danvers, MA) or control IgG (Santa Cruz) and 1 μg of Antibody Capture Affinity Ligand on a rocking platform overnight at 4°C. Eluted proteins were analyzed by western blot using anti-PTEN (A2B1) and anti-p53 (Cell Signaling Technology) antibodies as described (30).

Immunofluorescence

Cells were seeded on sterile 22 mm glass cover slides placed on a six-well plate and allowed to attach overnight. Cells were treated twice with GEN (40 nM or 2 μM, as indicated for each study) at $t = 0$ and 24 h, fixed and permeabilized in ice-cold methanol for 10 min. Immunofluorescence was done using the Vectastain *elite* ABC kit (Vector Laboratory, Burlingame, CA) as described (30). Antibodies used were: PTEN (1:200); phospho-PTEN (Ser 380) (1:200; Cell Signaling Technology) and p53 (1:500). Cells were mounted with Vectashield Mounting Medium with 4′,6-diamidino-2-phenylindole (nuclear stain) and analyzed for immunofluorescence under a Carl Zeiss Axiovision microscope (Carl Zeiss AG, Oberkochen, Germany). At least 500 cells were counted from five random areas per slide (×20 objective), with three slides for each treatment group.

Cell proliferation assay

Cell proliferation was evaluated using the 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide assay following the manufacturer's protocol (American Type Culture Collection). Cells were seeded in 96-well plates and treated with GEN (2 μM) or vehicle (dimethyl sulfoxide) every 2 days for 6 days. Absorbance values (570 nm) reflect the ability of metabolically active cells to reduce the yellow tetrazolium 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide salts into a purple precipitate.

Transient transfection and luciferase assays

The PTEN-luc reporter construct, human PTEN in pGL3b vector, was a gift from Dr Eileen D.Adamson [The Burnham Institute, La Jolla, CA (31)]. Cells were transfected using Lipofectamine 2000 (Invitrogen) with PTEN-luc reporter plasmid or empty (pGL3b) vector (each added at 0.2 μg/well) as described (32). After treatment with vehicle with and without added GEN (2 μM) post-transfection using either scrambled RNA (scRNA) or PTEN siRNAs (50 nM), cells were lysed in lysis buffer (Promega, Madison, WI), and quantitative determination of luciferase activity was carried out using a MLX Microplate Luminometer (Dynex Technologies, Chantilly, VA). *Renilla* luciferase (8 ng/well) activity was used as an internal control for transfection efficiency among cells and was measured using a Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was normalized to *Renilla* luciferase for each sample. Data are presented as means ± SEMs from three independent experiments performed in triplicate.

Chromatin immunoprecipitation assays

Cells were treated similarly as for the immunoprecipitation studies (above) and then processed for chromatin immunoprecipitation using the ChIP-IT Express Enzymatic Kit, following the manufacturer's recommendations (Active Motif, Carlsbad, CA). Polymerase chain reaction (PCR) with primers spanning the p53 binding sites on the PTEN promoter (33) (forward, 5′-CAAAAGCCGCAGCAAGTG-3′ and reverse, 5′-GAGCGCAGAGTCCCCAAG-3′; 115 bp) was carried out under the following conditions: hot start at 94°C for 5 min and then 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s with final extension at 72°C for 10 min. PCR products were resolved on a 3% agarose gel containing ethidium bromide.

Fluorescence-activated cell sorting

For each group, at least 10 000 cells were stained with propidium iodide and analyzed with a Becton Dickinson FACSCalibur. The proportion of cells in sub-G_1/G_0, G_1/G_0, S and G_2/M phases were determined with the Cell Quest software program (BD Biosciences, San Jose, CA).

Acini morphogenesis assay and image acquisition

MCF-10A cells were seeded on a layer of Matrigel (BD Biosciences) in eight-well chamber slides and allowed to form acini as described (29). Culture medium containing 2% charcoal-stripped horse serum and 5 ng/ml epidermal growth factor (EGF) without (vehicle alone) or with added GEN (2 μM) was refreshed every 4 days. Acini number and diameter were assessed at days 6 and 12 of culture using a phase contrast microscope (Carl Zeiss) (×20 objective). Indirect immunofluorescence of acinar structures was performed as described (29). The primary antibodies used were PTEN (1:200) and p53 (1:200). Confocal images were collected on a Zeiss LSM510 confocal microscope (×20 objective).

Data analysis

Statistical analyses was done using StatView version 5.0 for Windows. Data were analyzed using Student's *t*-test, one-way analysis of variance or two-way analysis of variance. Differences between means in two-way analysis of variance were further analyzed by Tukey's test. P values <0.05 were considered statistically significant.

Results

Expression of PTEN in MECs

We previously showed that prepubertal dietary exposure to SPI, the main component of infant soy formulas, and to CAS diet supplemented with the major soy isoflavone GEN protected against NMU-induced mammary tumors in rats, relative to the CAS group (11,12). Second to p53, PTEN is the most common tumor suppressor mutated or inactivated in human cancers including breast cancer (17). PTEN expression was assessed in MECs isolated from young adult rats at postnatal day 50 after lifetime exposure to CAS or SPI, by QPCR. Transcript levels of *PTEN* were increased in MECs of rats fed SPI relative to CAS (Figure 1a, left). To mechanistically dissect the functional implications of increased expression of *PTEN* in MECs *in vivo*, the effects of GEN were evaluated in the non-tumorigenic human mammary epithelial cell line, MCF-10A *in vitro*. The dose of 2 μM GEN is within the concentration range found in plasma of infants fed soy-based formulas (34) and of the Asian population with continuous exposure to GEN from daily soy consumption (35).

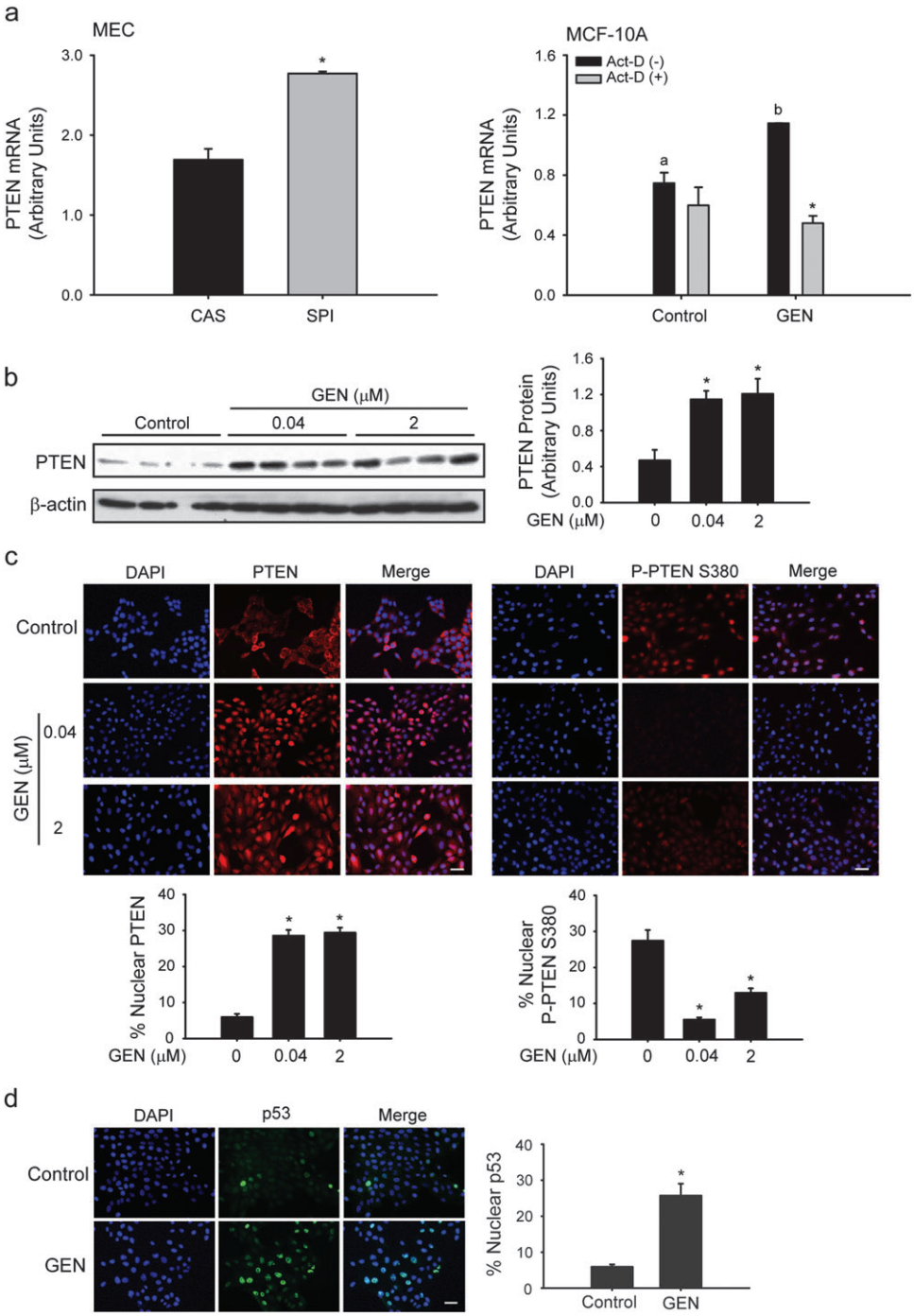

Fig. 1. GEN induces nuclear accumulation of PTEN and p53 in MECs. (**a**) 'Left', transcript levels of PTEN in MECs from rats fed CAS or SPI analyzed by QPCR. Values are means ± SEMs; $n = 4$ rats per diet group ($^{*}P < 0.05$ relative to CAS). 'Right', elevated *PTEN* gene expression after GEN (2 μM) treatment of non-malignant MCF-10A cells is due to increased transcription. Transcript levels were quantified by QPCR and normalized to 18S rRNA. Means with different letters differed at $P < 0.05$; $^{*}P < 0.05$ relative to absence of actinomycin-D (Act-D; 1 μg/ml) within each treatment group. (**b**) Western blot analysis of whole cell extracts from cells treated with GEN (40 nM and 2 μM). PTEN protein levels were compared with control (vehicle). Each lane represents an individual treatment sample and contains 50 μg of total protein. Immunoreactive bands were quantified by densitometric scanning and values normalized to those of loading control β-actin and are presented as histograms (right panel). (**c** and **d**) GEN increases the number of nuclear PTEN (red) and p53 (green)-positive MCF-10A cells, while decreasing nuclear accumulation of inactive Phospho-PTEN (Ser 380). Merge shows nuclear localization (4′,6-diamidino-2-phenylindole; blue); representative images for each group are shown from three independent experiments. Bar, 50 μM; $^{*}P < 0.05$ relative to control.

In addition, this dose is within the range found in the plasma of rats fed a lifetime diet containing SPI or GEN (36). MCF-10A, similar to normal human breast epithelial cells, are ER-negative (37) and unlike the tumorigenic mammary epithelial cell line MCF-7, do not undergo apoptosis with GEN treatment (15). MCF-10A treated with GEN for 6 h had higher transcript levels of PTEN compared with control (vehicle-treated) cells (Figure 1a, right). GEN induction of PTEN transcript levels was blocked by the transcriptional inhibitor actinomycin D (1 μg/ml) added 1 h prior to GEN treatment. Thus, induction of *PTEN* expression by dietary SPI *in vivo* was recapitulated by GEN *in vitro* and results in part, from transcriptional regulation of the *PTEN* gene.

GEN increases nuclear levels of PTEN and p53 in MCF-10A cells

PTEN function can be regulated by its subcellular localization, with normal cells preferentially showing nuclear PTEN localization (38). To determine the major site of action of GEN-induced PTEN in MCF-10A cells, PTEN protein levels were assessed by western analysis and immunofluorescence, in cells treated with GEN twice (at $t = 0$ and 24 h; sample collection at 24 h after last treatment) at 40 nM and 2 μM concentrations. We considered the lower dose of GEN to correspond to the serum concentrations of occasional soy consumers. PTEN protein levels were increased by 3-fold in whole extracts of cells treated with either dose of GEN, when compared with control cells (Figure 1b). Immunofluorescence demonstrated that GEN-treated cells had increased accumulation (by 6-fold) of nuclear PTEN (Figure 1c). The nuclear-localized PTEN is predominantly in the active form since parallel immunofluorescence using a specific antibody against inactive, phosphorylated-PTEN (P-PTEN S380) revealed significantly decreased levels of this protein with GEN treatment at either dose (Figure 1c). Interestingly, nuclear accumulation of p53 protein was significantly enhanced (by 5-fold) similar to PTEN, with GEN treatment (Figure 1d).

GEN increases nuclear colocalization and physical interaction of PTEN and p53 in vivo

p53 protein levels are reduced in PTEN-null cells and tissues, and reintroduction of wild-type or phosphatase-dead PTEN protein stabilizes p53 protein levels (26). To ascertain whether GEN-induced nuclear p53 levels are dependent on the nuclear levels of PTEN similarly elevated by GEN, cells were transfected with 50 nM of *PTEN* siRNA or scrambled (non-specific) scRNA, prior to vehicle or GEN (2 μM) treatment and then processed for dual immunofluorescence. Transient knockdown of *PTEN* by siRNA abolished basal and GEN-induced PTEN protein levels as shown in western blot, without any effect on loading control protein (Figure 2a). A dual immunofluorescence colocalization assay was performed to assess potential interactions between PTEN and p53 based on spatial localization. GEN treatment increased nuclear localization of PTEN and p53 in scRNA-transfected cells (Figure 2b). Treatment with GEN also resulted in increased nuclear colocalization of p53 and PTEN (Figure 2b; scRNA), consistent with PTEN–p53 complex formation predominantly occurring in the nucleus (26). However, not all nuclear-localized p53 or PTEN were found to colocalize (Figure 2b and c). In the presence of *PTEN* siRNA, nuclear p53 levels were significantly reduced in control and GEN-treated cells. To evaluate whether nuclear colocalized p53 and PTEN are physically associated, whole cell lysates from control and GEN-treated cells were immunoprecipitated with PTEN antibody or control IgG, and p53 and PTEN levels in immunoprecipitates were analyzed by western blots. p53 was co-immunoprecipitated with PTEN in GEN-treated cells but not in control (vehicle-treated) cells (Figure 2d), indicating GEN-mediated promotion of these proteins' physical association. Non-specific IgG did not immunoprecipitate either p53 nor PTEN protein in the same extracts, indicating specificity of the immune reactions.

GEN induction of PTEN promoter activity requires PTEN and p53

p53 can upregulate *PTEN* transcription through binding to p53 binding sites in the *PTEN* promoter (25) (Figure 3a). To determine whether GEN induction of nuclear PTEN leads to enhancement of *PTEN* transcriptional regulation involving p53, *PTEN* promoter activity was measured in control and GEN-treated cells by dual luciferase assay in the presence or absence of siRNAs targeting p53 or PTEN (Figure 3b and c). Transfection of PTEN-luc construct significantly increased luciferase activity in both control and GEN-treated cells relative to pGL3b vector-transfected cells. GEN treatment augmented *PTEN* promoter activity relative to control (vehicle) cells (by 1.5; $P = 0.031$). siRNA to *p53* decreased basal (55% inhibition) and GEN-induced (81% inhibition) *PTEN* promoter activity (Figure 3b). Interestingly, transient *PTEN* knockdown by siRNA similarly reduced *PTEN* promoter activity in control and GEN-treated cells (52 and 70% inhibition, respectively) (Figure 3c). GEN enhancement of *PTEN* promoter activity and the corresponding reductions in basal and GEN-induced activities with siRNAs to *p53* and *PTEN* were confirmed at the level of the PTEN messenger RNA by QPCR of transfected cells (Figure 3b and c). We next examined the effects of GEN on p53 and PTEN recruitment to the *PTEN* promoter by chromatin immunoprecipitation assay. Chromatin preparations isolated from control and GEN-treated cells were immunoprecipitated with either anti-p53, anti-PTEN or control IgG antibodies, and immunoprecipitated DNA was analyzed by PCR, using primer sets designed within the region of the *PTEN* promoter containing the p53 binding sites (Figure 3a). The recruitment of p53 to the *PTEN* promoter was enhanced by GEN, consistent with GEN regulation of *PTEN* transcription involving its induction of nuclear p53 levels (Figure 3d, top panel). GEN also increased the recruitment of PTEN to the p53 binding sites of the *PTEN* promoter (Figure 3d, middle panel). The amounts of PCR product with input DNA were comparable among samples (Figure 3d, bottom panel) and no PCR product was present in samples immunoprecipitated with control IgG, indicating antibody-specific immunoprecipitation procedures.

GEN antiproliferative effects are PTEN-dependent

While cytoplasmic PTEN decreases phospho-Akt levels and induces apoptosis, nuclear PTEN has been associated with G_0–G_1 cell cycle arrest by its downregulation of cyclin D1 (39). To evaluate if GEN induction of nuclear PTEN in MECs (Figures 1c and 2b) results in cell cycle arrest, MCF-10A cells were treated with vehicle or vehicle containing GEN (2 μM), and cell proliferation was measured after 6 days using the 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide assay. GEN modestly but significantly decreased cell proliferation; this effect was reversed in cells pretreated with *PTEN* siRNAs, showing PTEN dependence (Figure 4a). Since similar treatment with GEN did not change the number of viable cells nor result in induction of apoptosis (data not shown), decreased cell proliferation by GEN may reflect cell cycle arrest, a process attributed to nuclear PTEN (40). To determine whether increased levels of nuclear PTEN with GEN leads to G_0–G_1 arrest, cell cycle distribution was analyzed in control and GEN-treated cells by fluorescence-activated cell sorting analysis. GEN treatment increased the percentage of cells in G_0–G_1 phase of the cell cycle (56.72 ± 0.46 versus 62.97 ± 0.51; $P = 0.003$), whereas lowering that in the S phase (25.54 ± 0.06 versus 20.69 ± 0.05; $P = 0.005$) (Figure 4b). As a positive control for G_0–G_1 arrest (41), cells were administered growth media without added EGF for the same period as GEN (Figure 4b, no EGF). Absence of EGF treatment resulted in increased and decreased percentage of cells in the G_0–G_1 and S phases, respectively. Transcript levels of cyclin D1, a positive regulator of G_0/G_1–S transition and a nuclear PTEN target (42), and of pleiotrophin, a PTEN (negatively)-regulated growth factor (43), were evaluated in vehicle- and GEN-treated cells transfected with scRNAs or *PTEN* siRNAs, by QPCR. GEN treatment for 6 h decreased cyclin D1 (58% decrease; $P = 0.031$) and pleiotrophin (53% decrease; $P = 0.042$) transcript levels. *PTEN* siRNA addition abrogated GEN downregulation of these genes' expression (Figure 4c).

GEN promotes early lobuloalveolar differentiation of MCF-10A cells

Three-dimensional culture of MCF-10A cells in Matrigel constitutes an excellent model to study mammary differentiation (29). Single-seeded cells plated on Matrigel-coated chamber slides form acini with hollow lumens by day 10 of culture, resembling mammary gland morphology *in vivo*. To further determine the functional consequence of GEN promotion of PTEN expression and of PTEN–p53 nuclear colocalization in MCF-10A cells, the ability of GEN to induce acini formation was assessed. Cells without or with added GEN (2 μM) were evaluated for acini morphogenesis at days 6 and 12, periods corresponding to pre- and post-differentiated states, respectively (29). At day 6, a significant increase in the numbers of acini in the 60–100 ($\times 10^2$) μm^2 range was observed with GEN treatment, indicative of early lobuloalveolar differentiation (Figure 5a and b). The numbers of acini with increasing sizes [(100–140, >140) ($\times 10^2$)] continued to increase at day 12, albeit

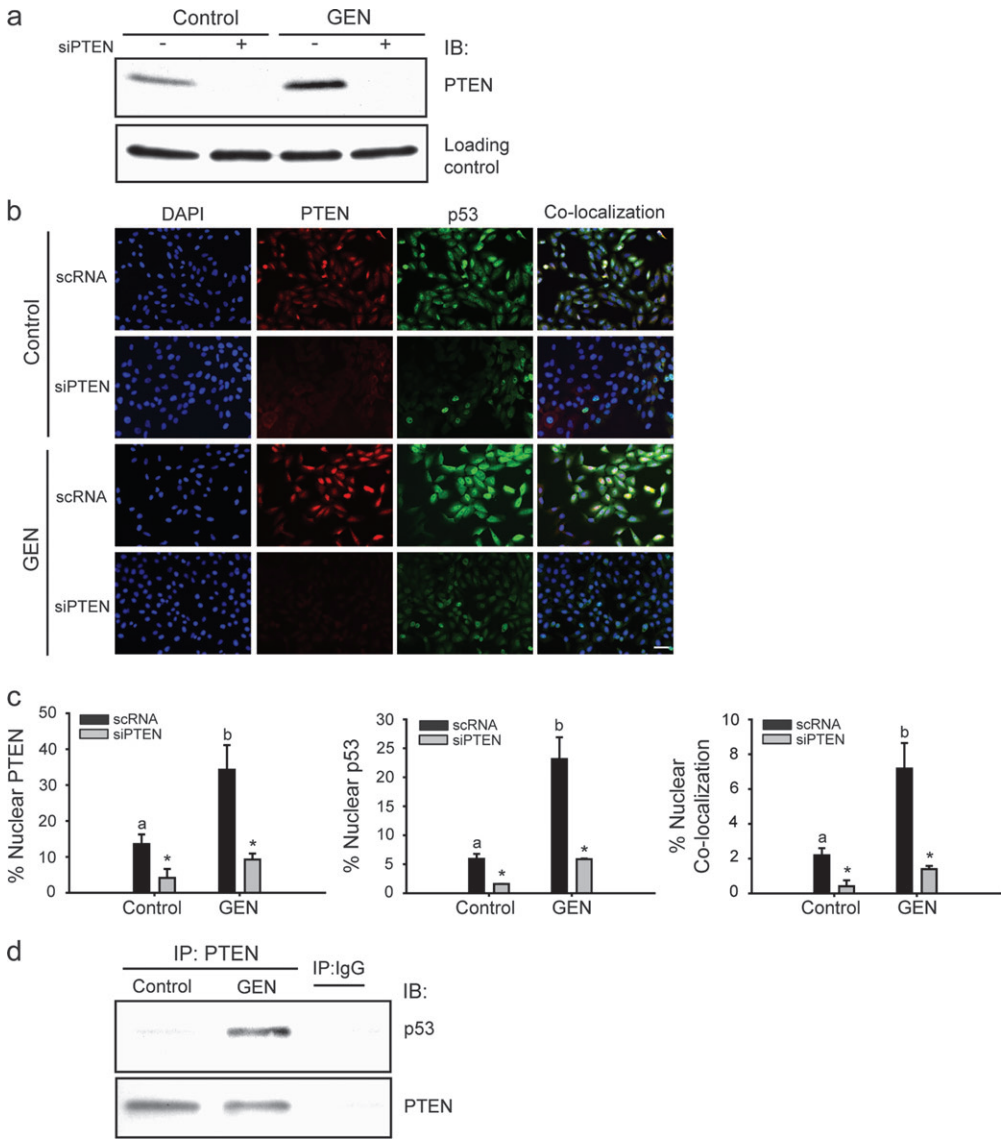

Fig. 2. GEN increases colocalization of nuclear PTEN and p53 and their physical interaction *in vivo*. (**a**) siRNAs targeting *PTEN* abolish basal and GEN-induced PTEN protein levels; loading control is a non-specific protein (140 kDa) present in the same blot. (**b**) GEN increases nuclear levels of PTEN and p53 in MCF-10A cells and their subsequent colocalization. Cells were immunostained for PTEN (red) and p53 (green) before being counterstained for 4′,6-diamidino-2-phenylindole (blue). Colocalization of anti-PTEN and p53-stained cells coincide with 4′,6-diamidino-2-phenylindole and show an orange color. Representative images for each group are shown from three independent experiments; bar, 50 μM. (**c**) The percentage of nuclear PTEN, p53 and colocalized PTEN and p53 was calculated by counting five random areas per slide with three slides for each group. Means with different letters (a,b) differed at $P < 0.05$; $^{*}P < 0.05$ relative to non-targeting scRNA within each group. (**d**) Cell lysates from MCF-10A cells were immunoprecipitated with mouse monoclonal PTEN antibody, followed by immunoblotting with anti-p53 or anti-PTEN antibodies. Representative blots from two independent experiments with same results are shown.

GEN effects over control only tended to be significant (Figure 5b). PTEN and p53 protein localization/colocalization in acinar structures (day 6) were evaluated by immunofluorescence and confocal imaging. Nuclear PTEN and p53 immunoreactivities were higher in GEN-treated acini relative to corresponding controls (vehicle treated), and a number of these acini showed PTEN–p53 colocalization (Figure 5c and d), similar to the results for GEN-treated cells grown in monolayers (Figure 2b). These results are consistent with the involvement of PTEN and p53 in GEN-induced early lobuloalveolar differentiation of MECs, supporting a recent observation that PTEN regulates acini morphogenesis of MECs (44).

Discussion

In this study, we establish induction of nuclear PTEN–p53 cross-regulation by GEN in MECs as a novel mechanism of breast cancer prevention by dietary factors. We provide evidence to support a model in which GEN induces an autoregulatory loop between PTEN and p53 to promote mammary epithelial cell cycle arrest and early lobuloalveolar differentiation (Figure 6). GEN induction of PTEN expression and nuclear accumulation elicits a sequence of PTEN-dependent events as follows: (i) increased nuclear p53 accumulation; (ii) enhanced PTEN–p53 physical interaction; (iii) increased recruitment of the PTEN–p53 complex to the p53 binding sites of the *PTEN* promoter; (iv) higher *PTEN* promoter activity and (v) promotion of cell cycle arrest and lobuloalveolar differentiation and attenuated expression of proliferative genes *cyclin D1* and *pleiotrophin*. Taken together, our findings identify PTEN pathway activation involving p53 as an early molecular event mediating dietary factor effects in the prevention of mammary tumorigenesis.

Around 40% of breast cancer cases are preventable by a healthy diet, exercise and weight control alone (45). While it is widely accepted that early (prepubertal) consumption of soy foods is associated with a decreased risk of breast cancer (7), mechanisms underlying

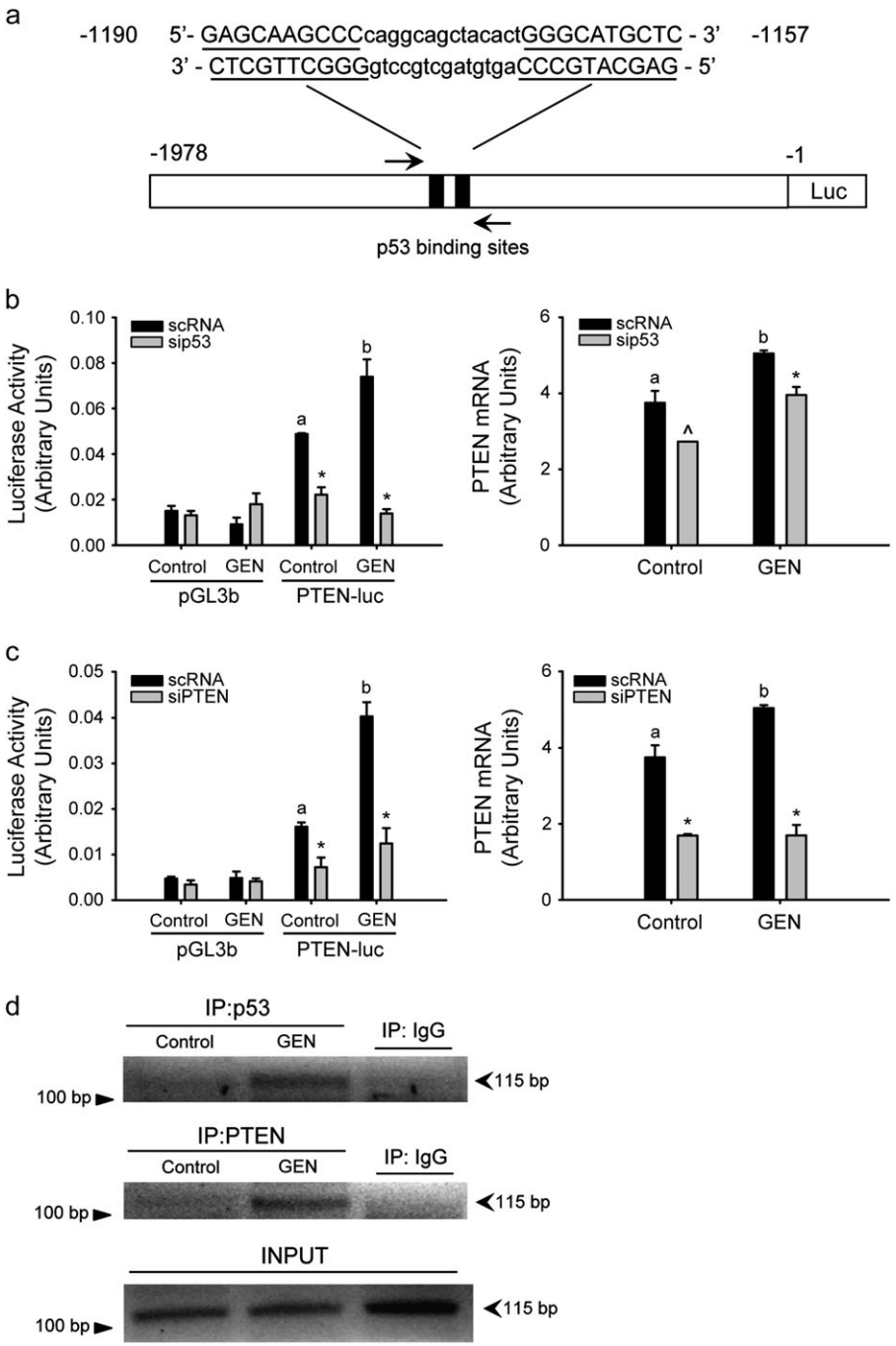

Fig. 3. Induction of *PTEN* promoter by GEN requires PTEN and p53. (**a**) Schematic representation of the PTEN-luc reporter construct used for luciferase assay containing the two p53 binding sites (black box). MCF-10A cells were cotransfected with 0.2 μg of *PTEN* promoter–reporter construct (PTEN-luc) or empty vector pGL3-basic vector (pGL3b) and control siRNA (scRNA) or siRNA targeting *p53* (**b**) or *PTEN* (**c**). Following overnight serum starvation, cells were treated twice with GEN for 24 h each and then luciferase activity was determined (Materials and Methods). Each siRNA panel represents a separate experiment repeated twice with similar results. *PTEN* messenger RNA levels were determined under similar treatment conditions and quantified by QPCR. Means with different letters (a,b) differed at $P < 0.05$; $^{*}P < 0.05$ and $^{\wedge}P = 0.085$ relative to non-targeting scRNA within each group. (**d**) Cross-linked sheared chromatin was immunoprecipitated with antibody against p53 or PTEN. After reversal of cross-links, DNA was analyzed by PCR using primers that amplify the *PTEN* promoter region containing the p53 binding sites.

dietary protection against mammary tumorigenesis remain poorly understood. Here, we provide strong support for a molecular mechanism involving increased PTEN expression in MECs to underlie mammary tumor protection by diet. The non-tumorigenic, classical ER-negative, human mammary epithelial cell line, MCF-10A, expresses both wild-type PTEN and p53 and represents an ideal *in vitro* system that mimics prepubertal (estrogen insensitive) mammary gland (46). The current study used GEN at physiologically relevant concentrations mimicking those found in plasma of human subjects regularly consuming soy foods (35). Our results effectively preclude increased DNA damage and apoptosis as underlying the GEN-mediated molecular changes reported here since higher doses of GEN than used in the present study were found to be non-genotoxic to MECs (47).

Although PTEN is known as a potent inhibitor of the PI3K–Akt pathway, recent emerging data support phosphatase-independent and cellular localization-dependent roles of PTEN. For example, while

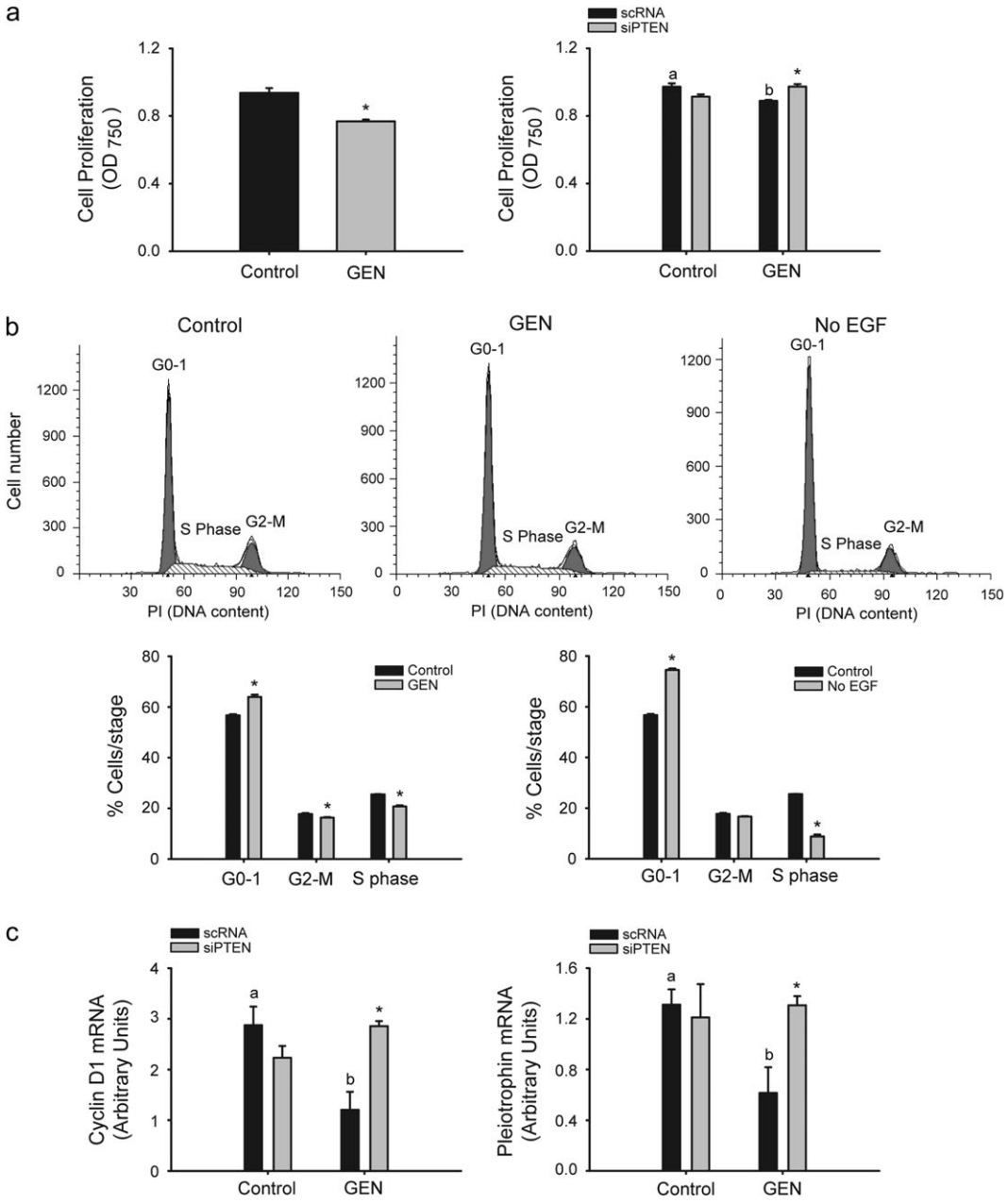

Fig. 4. The anti-proliferative effect of GEN requires PTEN. (**a**) Decreased cell proliferation by GEN in a PTEN-dependent manner in MCF-10A cells measured by the 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide assay after 6 days of GEN treatment. (**b**) Cell cycle distribution determined by fluorescence-activated cell sorting analysis shows G_0–G_1 arrest and lower percentage of cells in the S phase after GEN treatment. As a positive control for G_0–G_1 arrest, cells were administered media in the absence of EGF and analyzed at the same time. Values are means ± SEMs from two independent experiments performed with triplicate samples. $^*P < 0.05$ relative to control for each stage of cell cycle. (**c**) Transcript levels of the positive regulator of G_1–S transition, cyclin D1 and the proliferative growth factor pleiotrophin are decreased by GEN in a PTEN-dependent manner. Means with different letters (a,b) differed at $P < 0.05$; $^*P<0.05$ relative to non-targeting scRNA within each treatment group.

cytoplasmic PTEN is considered to mediate apoptosis, nuclear PTEN plays a key role in chromosomal integrity, cell cycle arrest and differentiation (42). Loss of nuclear PTEN has been correlated with decreased differentiation of normal cells, favoring neoplastic transformation (48). We demonstrate here that PTEN is both a target and regulator of p53 action, leading to increased MEC differentiation. The GEN-induced PTEN autoregulatory loop is initiated by increased nuclear localization of PTEN, which promotes nuclear retention of p53 and the subsequent transactivation by the PTEN–p53 complex of the *PTEN* promoter. Several features of this novel mechanism are noteworthy. First, GEN effects on p53 largely occur through PTEN, as demonstrated by the highly significant PTEN-mediated increase in p53 nuclear localization in the relative absence of GEN effects on p53 expression (data not shown). These results are consistent with the observed lack of apoptosis in GEN-treated MCF10A cells (undetectable sub-G_0/G_1 population), which would be predicted otherwise if GEN leads to increased DNA damage concomitant with elevated p53 expression. Second, while p53 is known to transactivate the *PTEN* gene (25) and that PTEN has been demonstrated to regulate p53 levels in both phosphatase-dependent and -independent manners (26,49), the present results to the best of our knowledge, constitute the first report of a functional PTEN–p53 transcriptional complex underlying increased *PTEN* promoter activity in MECs. The latter is consistent with the findings that PTEN and p53 mutations are mutually exclusive

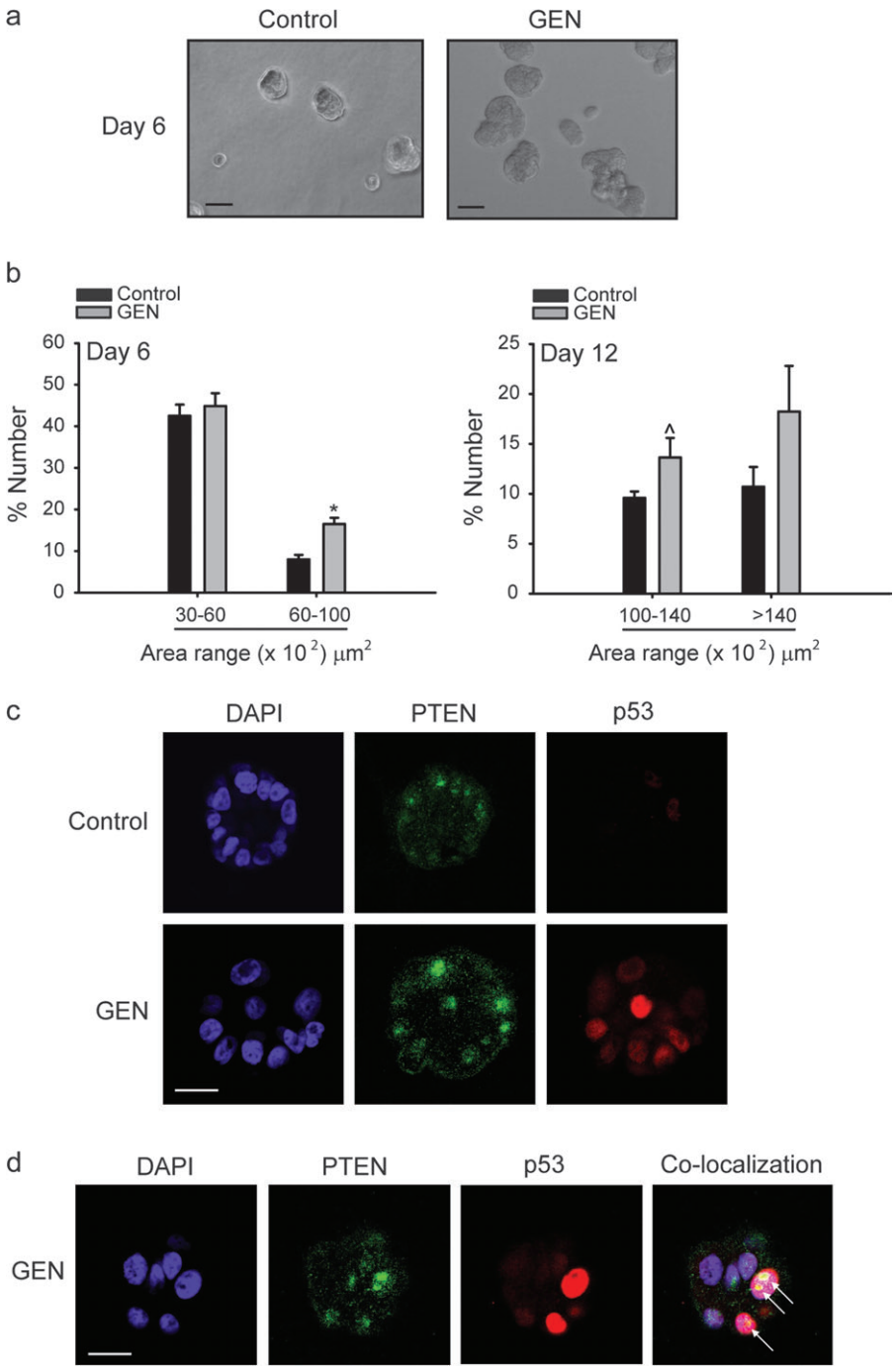

Fig. 5. GEN promotes early lobuloalveolar differentiation. (**a**) MCF-10A cells were cultured in Matrigel to allow acini formation as described in Materials and Methods. The numbers of acini formed were counted at days 6 and 12 of mammary acini morphogenesis from three random areas per chamber with four chambers per treatment group; bar, 50 μM. (**b**) Results are mean ± SEM from two independent experiments. $^{*}P < 0.05$ relative to control. (**c**) Increased nuclear PTEN and p53 in GEN-treated MCF-10A acini at day 6. Subcellular localization of PTEN (green) and p53 (red) was recorded by confocal microscopy. Representative images are from four independent chambers for Control or GEN; bar, 20 μM. (**d**) Z-section showing colocalization of PTEN and p53 (arrow) in GEN-treated cells at day 6; bar, 20 μM.

in human breast cancers (50), suggesting that loss of either tumor suppressor is sufficient to facilitate tumorigenesis, partly due to the eventual loss of PTEN expression. Finally, while the initiation of the PTEN autoregulatory loop by GEN leads to a significant increase in cell cycle arrest and promotion of early lobuloalveolar differentiation, the biological changes are modest, suggesting multiple pathways underlying dietary effects against tumorigenesis. In agreement with this, we (14,51) and others (52) have identified numerous genes and signaling pathways that are regulated by dietary factors in a variety of tissues and cell types.

Our work links the two most common tumor suppressor pathways, namely PTEN and p53, in the context of breast cancer prevention by soy isoflavone GEN. Albeit the mechanism for induction of PTEN levels by GEN is currently unknown, it is possible that GEN may be acting through classical ER-independent mechanisms via G protein-coupled receptor 30, a membrane receptor for estrogen (53), based on

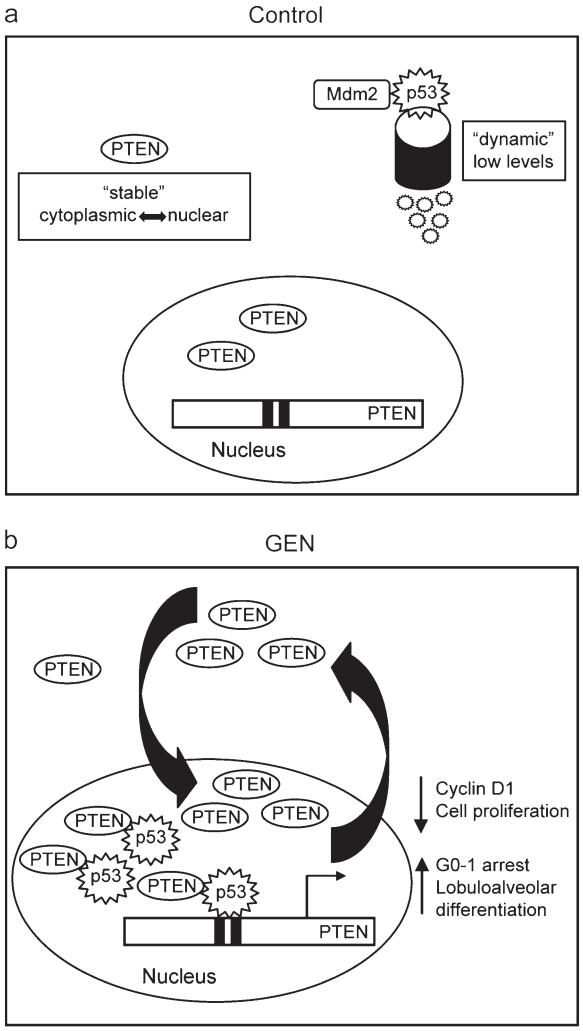

Fig. 6. Proposed model for GEN-induced nuclear PTEN–p53 cross talk in MECs to enhance PTEN signaling and subsequent MEC differentiation. (**a**) PTEN is constitutively expressed in resting cells and its function is regulated in part by subcellular localization, with nuclear preference in non-malignant cells. On the other hand, p53 is a dynamic protein that is maintained at low levels under normal conditions by Mdm2 degradation. (**b**) In the presence of GEN, the nuclear pool of PTEN is increased leading to increased levels of nuclear p53, promotion of PTEN–p53 complex formation, increased recruitment of the PTEN–p53 complex to the PTEN promoter, and hence, activation of PTEN transcription. A functional outcome of increased nuclear PTEN is the reduction in the expression of pro-proliferative genes *cyclin D1* and *pleiotrophin*, resulting in G_0–G_1 arrest and leading to early lobuloalveolar differentiation.

our preliminary results indicating GEN upregulation of G protein-coupled receptor 30 expression in MECs (data not shown). Our studies offer a mechanistic basis to target the PTEN activation pathway by the identification of dietary components with PTEN-promoting activity as a preventative strategy to reduce breast cancer risk.

Funding

United States Department of Agriculture (CRIS 6251-5100002-06S, Arkansas Children's Nutrition Center) to R.C.M.S.; Children's University Medical Group award to R.C.M.S. University of Arkansas Graduate Student Research Fund, Arkansas Children's Hospital Research Institute Student and Clinical Staff Research Intramural Grant to O.R. Pre-doctoral fellowship from the Department of Defense Breast Cancer Research Program (W81XWH-10-1-0047) to O.R.

Acknowledgements

We thank Dr Frank A. Simmen, Dr Shanmugam Nagarajan and laboratory members for helpful discussions and critical reading of this manuscript. We also acknowledge the use of the Confocal Microscopy Laboratory, University of Arkansas for Medical Sciences (Supported by National Institutes of Health Grants P20 RR16460; Dr Larry Cornett, Principal Investigator and NCCR-S10RR19395; Dr Richard Kurten, Principal Investigator).

Conflict of Interest Statement: None declared.

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Received February 28, 2010; revised June 7, 2010; accepted June 10, 2010

Available online at www.sciencedirect.com
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Journal of Nutritional Biochemistry 22 (2011) 605–611
Journal of Nutritional Biochemistry

REVIEWS: CURRENT TOPICS

Bidirectional signaling of mammary epithelium and stroma: implications for breast cancer—preventive actions of dietary factors

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Received 26 April 2010; accepted 18 September 2010

Abstract

The mammary gland is composed of two major cellular compartments: a highly dynamic epithelium that undergoes cycles of proliferation, differentiation and apoptosis in response to local and endocrine signals and the underlying stroma comprised of fibroblasts, endothelial cells and adipocytes, which collectively form the mammary fat pad. Breast cancer originates from subversions of normal growth regulatory pathways in mammary epithelial cells due to genetic mutations and epigenetic modifications in tumor suppressors, oncogenes and DNA repair genes. Diet is considered a highly modifiable determinant of breast cancer risk; thus, considerable efforts are focused on understanding how certain dietary factors may promote resistance of mammary epithelial cells to growth dysregulation. The recent indications that stromal cells contribute to the maintenance of the mammary epithelial 'niche' and the increasing appreciation for adipose tissue as an endocrine organ with a complex secretome have led to the novel paradigm that the mammary stromal compartment is itself a relevant target of bioactive dietary factors. In this review, we address the potential influence of dietary factors on mammary epithelial–stromal bidirectional signaling to provide mechanistic insights into how dietary factors may promote early mammary epithelial differentiation to decrease adult breast cancer risk.

Keywords: Mammary gland; Epithelium; Adipocyte; Diet; Breast cancer; Obesity

1. Introduction

Breast cancer is the most commonly diagnosed cancer and the second leading cause of cancer deaths among women in the United States. In 2009 alone, more than 190,000 new cases of invasive breast cancer were reported, which accounted for ~25% of all cancers among women in the United States [1]. Similar to all cancers, breast cancer is a genetic and epigenetic disease with diverse histopathological and clinical outcomes [2]. Although the major reasons for breast cancer deaths are complications arising from metastasis, the natural history of breast cancer involves progression through defined molecular, pathological and clinical stages [3,4]. The widely accepted view of breast tumor progression, known as linear progression [5], assumes the gradual transition of breast lesions from premalignant, hyperplastic states into ductal carcinoma *in situ*, invasive carcinoma and, finally, metastatic disease [6]. Recent clinical studies demonstrating heterogeneity in tumors from breast cancer patients now suggest that the linear progression model maybe overly simplistic [7,8]. In the more recently described diversity evolution model [9], the constant selection pressures provided by numerous environmental cues or therapeutic interventions are posited to lead to the high clonal diversity found in tumors as well as the drug resistance that may develop during treatment [10].

The mammary gland is comprised of myoepithelial and luminal epithelial cells embedded in a complex stromal matrix ('mammary fat pad') comprised predominantly of fibroblasts, adipocytes and macrophages (Fig. 1). The prevailing concept in the field is that the discrete mammary epithelial subtypes and neighboring stromal cells arise, respectively, from the asymmetric division of epithelial and mesenchymal cells of origin ('stem cells') and the subsequent differentiation of lineage-committed progenitor cells [11,12]. Emerging data on mammary stem cells have raised the possibility that this epithelial subpopulation 'sitting at the top' of the mammary epithelial hierarchy serves as initial target of oncogenic agents [11].

The transformation of normal mammary epithelial cells to malignancy is manifested as aberrant growth and survival responses to extracellular signals. The latter include those derived from the endocrine milieu, as well as from the stroma, whose physical proximity to epithelial cells allows for dynamic paracrine regulation and the integration of signals from circulating hormones and growth factors [13,14]. In a recent review, Arendt et al. [15] detailed the

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doi:10.1016/j.jnutbio.2010.09.008

complex local and systemic contributions of the stromal compartment to normal mammary development and to malignant breast development. Molecular and phenotypic changes within the stroma affect their interactions with neighboring cells, resulting in a microenvironment that can be supportive of epithelial progression to malignancy [16–18]. The distinct molecular signatures displayed by enriched populations of stromal cells underlying epithelial cell populations from normal breast tissue and invasive cancer [19,20] provide a convincing molecular rationale for the stromal compartment as instrumental to tumor progression. Increased understanding of the contribution of underlying stroma to breast cancer, predominantly an epithelial cell phenomenon, provides exciting potential for manipulating the mammary stromal compartment in the development of therapy [15,21]. Given the emerging evidence for dietary contribution to breast cancer risk [22] through diet-mediated regulation of mammary epithelial differentiation, proliferation and apoptosis [23–27] coupled with the recognition that mammary fate and ductal development are controlled to a large extent by mammary fibroblastic and adipocyte mesenchyme [15], the prospect that diet-associated components may equally influence mammary stromal biology to influence the course of differentiation or neoplastic growth of the mammary epithelium is not far-fetched.

The invitation to write this minireview was prompted by our findings that mammary stromal adipocytes are early biological targets of dietary factors, specifically of the major isoflavone genistein (GEN) *in vivo* [27]. In that report, we showed that limited exposure (i.e., *in utero* and lactational only) of female rat offspring to a maternal diet containing soy protein isolate (SPI) as major protein source resulted in mammary stromal adipocyte-specific genomic changes (e.g., lipogenic gene expression) coincident with increased differentiation of mammary tissues that were distinct from those exposed to the control diet with casein as the major protein source. Further, we showed that the functional consequence of SPI-mediated adipocyte metabolic changes on neighboring mammary epithelium *in vivo* can be recapitulated by GEN *in vitro* through direct actions on differentiated 3T3-L1 adipocytes, a function likely related to their increased secretion of the adipokine adiponectin with GEN treatment [27]. Little is known of the gene pathways and mechanisms by which specific dietary factors may target the stromal compartment to promote breast health. We begin this review by highlighting seminal information on cell signaling mechanisms underlying mammary tumor protection by dietary factors. Next, we describe how mammary stromal remodeling has been implicated in underlying epithelial biology, with a focus on the emerging links between mammary adiposity and mammary ductal development as an indication of adipose-directed signaling. Finally, we discuss recently described, albeit limited, information on stromal-localized molecular targets of dietary factors, which may serve as paracrine mediators of dietary factor action on mammary epithelial cells.

2. Dietary factors and mammary epithelial targets in breast cancer protection

The incidence of breast cancer is high in the United States [1], with an increasing trend noted globally [28], yet strategies addressing its prevention remain extremely limited. Indeed, the current emphasis on the clinical management and treatment of breast cancer dramatically contrasts with the inadequacy of efforts directed toward disease prevention. In addition, there is reluctance among the general populace to embrace the concept that nutrition and lifestyle constitute highly modifiable risk factors for the prevention of breast cancer. In part, this may be due to the oftentimes conflicting reports, based largely on epidemiological studies, of the protective health benefits of specific diets. For example, high dietary fat intake, especially high polyunsaturated fatty acids, has been linked to the promotion of breast cancer in animal models [29,30] but currently not in humans [31,32]. On the other hand, saturated fat consumption is linked to breast cancer in women, but this has not been conclusively demonstrated in animal studies [33]. Similarly, dietary vitamin A, carotenoid and Vitamin D intake has been individually shown to prevent breast cancer in a number of human and animal studies, although a unifying outcome remains lacking [34,35]. The differences in physiological status of human subjects (prepubertal and post-pubertal; premenopausal and postmenopausal), source of dietary factors (from foods or supplements) as well as varying doses and 'developmental window' of dietary exposure in the many studies described in the literature [22,32,36] had preempted conclusive indications of the breast cancer-preventive benefits of consumption of any dietary factor. While studies with animal models and cell lines have been faulted for their simplistic approach toward understanding dietary prevention of breast cancer susceptibility, given the heterogeneity of the human population, these models have been invaluable in providing mechanistic insights regarding the contributions of specific bioactive components to breast cancer risk.

Efforts to understand the mechanisms underlying the breast cancer-preventive effects of dietary factors have focused on their

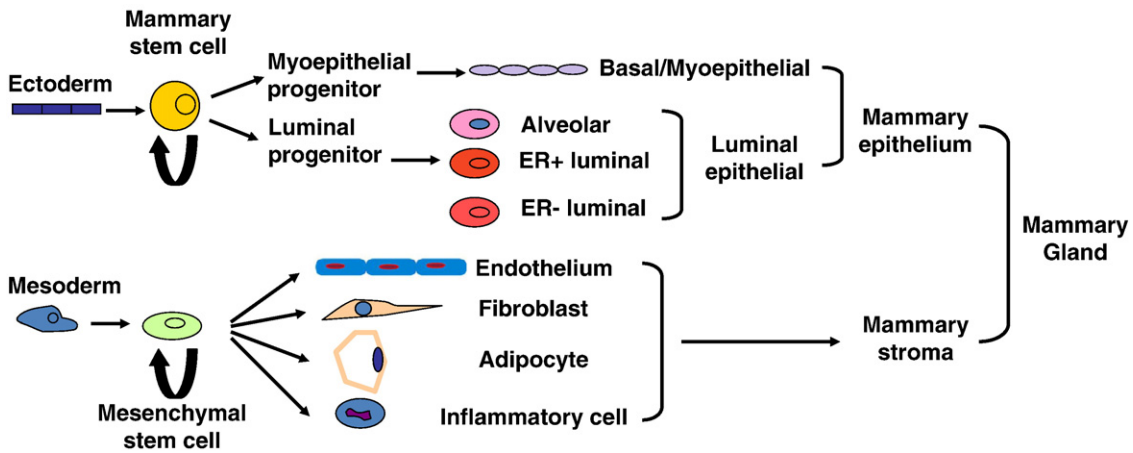

Fig. 1. The origin and lineage of the different cell types in the mammary gland. The mammary epithelium (luminal and myoepithelial) is embedded in the complex stromal matrix (also designated mammary fat pad) composed predominantly of fibroblasts, adipocytes and immune cells. The complexity of the mammary gland is a function of its distinct constituent cell types, which are subject to different endocrine and local regulation and which exhibit diverse functions. ER+ve, estrogen receptor positive; ER−ve, estrogen receptor negative.

biological and genomic consequences on mammary epithelial cells, where breast cancer arises. In particular, curcumin from turmeric [37], resveratrol from grape [38], capsaicin from chili pepper [39], flavonoids such as hesperetin and naringenin in citrus fruits and tomatoes [40], isoflavones (e.g., GEN, daidzein) from legumes and red clover [41,42] and epigallocatechin-3-gallate from green tea [43] have been demonstrated to provide different levels of preventive effects in rodent and cell culture models. An extensive discussion of the literature on the numerous mechanisms reported to underlie dietary prevention of breast cancer is beyond the scope of this current review, given the excellent recent reviews on this subject [44–48]. Suffice it to say that common mechanisms of actions have emerged: these include carcinogen activation/detoxification by metabolic enzymes, increased antioxidant and anti-inflammatory effects, induction of cell cycle arrest and inhibition of cell proliferation, decreased cell survival, enhancement of differentiation, increased expression and functional activation of various genes and corresponding proteins that are involved in DNA damage repair, tumor suppression and angiogenesis and down-regulation of oncogenes. Importantly, while the signaling pathways affected by various dietary factors in mammary epithelial cells are numerous, these pathways are interrelated, not mutually exclusive and as expected, utilize similar sets of genes previously elaborated in other tumor types [49].

Global gene expression profiling of mammary epithelial cells and subsequent functional annotation of gene expression changes have proven to be an effective tool for the discovery of novel pathways mediating dietary factor protection of mammary tumorigenesis. In studies from our laboratory using Affymetrix GeneChip microarrays [50], we showed a very low percentage of epithelial genes (~0.5% of 14,000 genes evaluated) whose expression is altered by exposure to either SPI or GEN diet beginning *in utero* to early adult stage (postnatal day 50), relative to control casein diet. The functional association of these identified genes with signaling pathways involved in immune response, protein and carbohydrate metabolism, growth regulation and stem cell niche (e.g., Wnt and Notch pathways) has provided invaluable insights into important targets of SPI-associated bioactive components and, in particular, GEN to induce epithelial changes for increased resistance to carcinogenic agents [51,52]. Indeed, our independent identification of the tumor suppressor *PTEN* [53] and of E-cadherin/Wnt/β-catenin signaling [54] as molecular pathways influenced by dietary exposure to SPI and GEN *in vivo* and by GEN *in vitro* has been bolstered by the recently elaborated linkage between these two signaling pathways in the regulation of normal and malignant mammary stem/progenitor cells *in vivo* and *in vitro* [55]. Similar support has been provided by other published studies, including those for epigallocatechin-3-gallate [56], phytoestrogens [57] and polyunsaturated fatty acids [58]. Taken together, the cellular pathways mediating dietary factor actions in the context of mammary epithelial growth regulation implicate their collective opposing actions on the expression and/or activity of tumor suppressors and oncogenes and their respective downstream targets.

3. Mammary stromal signaling in breast cancer prevention

How does the mammary stroma compartment potentiate resistance of its neighboring preneoplastic cells to tumor-initiating events? Much insight has emerged from studies on carcinoma-associated stromal fibroblasts, which can transdifferentiate into myofibroblasts and which have been demonstrated to promote primary tumor growth in human xenograft models when compared to noncancerous stromas [19,20]. The altered activity of tumor-associated stromal fibroblastic cells was associated with genetic and epigenetic alterations in specific gene subsets including that of the tumor suppressor *p53*, leading to increased expression of growth factors, cytokines and extracellular matrix components and which, by paracrine signaling, promoted neoangiogenesis and epithelial-to-mesenchymal transition in neighboring cells [19,59]. In an elegant recent study by Trimboli et al. [60], the conditional inactivation of the tumor suppressor *PTEN* in stromal fibroblasts of mouse mammary glands was shown to promote the initiation, progression and malignant transformation of mammary epithelium. *PTEN* loss was linked to increased extracellular matrix component deposition and innate immune infiltration, two key events associated with tumor malignancy and with activation of Ras, JNK and Akt growth-regulatory pathways [60]. This and similar studies [61–63] strongly support the notion that altered signaling in the tumor stroma, in this case, stromal fibroblasts, elicits aberrant epithelial growth regulation, leading to tumor manifestation.

Adipocytes constitute a significant component of the mammary stromal compartment and, similar to fibroblasts, are considered essential for mammary tumor growth and survival. While the mouse mammary fat pad consists primarily of adipocytes, this is not the case for the human mammary gland, where the developing mammary epithelium is closely sheathed by stromal fibroblasts. Nevertheless, the proximity of adipocytes to the epithelium and their high secretome activity [64,65] suggest significant influence. Indeed, the findings that (1) obesity, a disorder arising from altered gene–nutrient interactions, is a risk factor for breast cancer development [66], (2) diet-induced obesity in mice results in enlarged mammary glands and suppression of normal ductal development [67], and (3) adipose tissue from obese human subjects synthesize high and low levels of the adipokines leptin and adiponectin, respectively [68,69], which display opposing effects (promotion by leptin; inhibition by adiponectin) on mammary epithelial proliferation and which have been associated with regulation of mammary tumor development in mice [70], provide strong support for the influence of mammary adipocytes on breast cancer progression.

Interestingly, despite the increasing focus on obesity and nutrition/diet as major determinants of mammary epithelial oncogenesis, the connection between dietary factors with putative mammary tumor-protective effects and normal mammary adipose tissue biology has not been directly demonstrated. Two studies have recently appeared that highlight this association, albeit indirectly. Cho et al. [71] reported that the polyphenol (−)-catechin, among the many polyphenols present in green tea, enhanced the expression and secretion of adiponectin in 3T3-L1 adipocytes *in vitro*. The increase in adiponectin secretion by (−)-catechin was accompanied by increased insulin-dependent glucose uptake in differentiated adipocytes and decreased expression of the transcription factor Kruppel-like 7, which inhibits adiponectin expression [71]. While these *in vitro* findings did not directly address the consequence(s) of (−)-catechin promotion of adiponectin expression and secretion on mammary epithelial growth regulation, they are consistent with previous indications that green tea extracts have antiobesogenic activity [72] and inhibit mammary tumor initiation and progression in animal models of breast cancer [73]. In the second study by our group [27], we incorporated *in vivo* and *in vitro* strategies to link genomic and functional consequences in rat mammary glands upon *in utero*/lactational exposure to dietary SPI with paracrine signals from GEN-treated 3T3-L1 adipocytes to induce mammary epithelial differentiation. While our studies did not identify the paracrine signal(s) mediating the enhanced differentiation of mammary epithelial cells, we posited that one likely candidate is adiponectin, given the increased secretion of this adipokine in differentiated adipocytes treated with GEN at physiological doses [27]. Preliminary findings provide support to the latter, based on the higher adiponectin protein levels in the mammary glands of young adult female rat offspring exposed to SPI following the above dietary regimen, in the absence of changes in systemic levels of this adipokine (O. Rahal and R.C.M. Simmen, unpublished observations). Given that early only and

lifelong exposure to soy-enriched diets are mammary tumor-preventive in rodent models of carcinogenesis [52,74], findings that were borne out by epidemiological studies [75], the 'chicken-or-the-egg" question as to which mammary compartment (stromal or epithelial) is initially targeted by dietary factors to achieve the final outcome of increased mammary epithelial differentiation for decreased sensitivity to oncogenic agents, may constitute a fruitful direction for future investigation.

While the aforementioned studies investigated aspects of dietary influences on lipogenic and adipogenic regulators in the mammary adipocyte, mechanisms for dietary regulation at the level of adipocyte differentiation are also plausible. A great deal of our understanding of the molecular basis of adipocyte differentiation has been gained from studies of clonal fibroblastic preadipocyte cell lines (3T3-L1, 3T3-442A) and *ex vivo* studies of stromal vascular cells isolated from animals [76,77]. Committed preadipocytes, upon hormonal induction *in vitro* and via elusive *in vivo* signals, begin the differentiation program involving CREB-mediated phosphorylation of the transcription factor CAAAT-enhancer binding protein-β [77–79], followed by mitotic clonal expansion and activation of CAAAT-enhancer binding protein-α and peroxisome proliferator-activated receptor (PPAR)-γ. These, along with the sterol regulatory element binding protein-1c, transactivate a number of adipocyte-specific genes that maintain the adipocyte phenotype [80,81]. Throughout life, adipose tissue mass is regulated by a balance between formation (via hypertrophy of existing adipocytes and hyperplasia) and lipolysis. While the molecular events underlying adipocyte differentiation from precursor cells have been extensively studied, the precise origins of the adipose tissue *in vivo* are still poorly understood. In this context, two important recent advances in our understanding are noteworthy. First, using novel PPAR-γ reporter mouse strains (PPAR-γ-Rosa26 reporter and PPAR-γ-TRE-H2B-GFP) where endogenous PPAR-γ promoter leads to indelible marking of daughter cells with LacZ or GFP, Tang et al. [82], performed cell lineage tracing experiments. These elegant studies revealed that most adipocytes reside in the mural cell compartment in close proximity to the adipose vasculature and are already committed to an adipocyte fate *in utero* or early postnatal life. The second major advance in this area has been the identification of early adipocyte progenitor cells in the adipose tissue using flow cytometry. Using fluorescence-activated cell sorting, Rodeheffer et al. [83] identified cells that are $Lin^{-}CD29^{+}CD34^{+}Sca1^{+}CD24^{+}$ residing in the adipose tissue and that likely represent early adipocyte precursors since they can reconstitute a normal adipose tissue when injected into 'fat-less' lipodystrophic mice. It should be noted that the origin of adipocytes in the mammary fat pad has not been examined to date. In light of these studies, it is important to begin to address whether diet/dietary factor-associated cancer protection may be linked with altered commitment/differentiation of mammary preadipocytes.

4. Dietary factors and candidate mammary stromal targets for breast cancer prevention

While there is a paucity of information to directly link the targeting of specific mammary stromal cell types by known dietary factors to neighboring mammary epithelial growth regulation, a few candidate mediators have emerged. The most relevant are the adipokines adiponectin and leptin, which, because of their mammary adipocyte source, demonstrated regulation of mammary epithelial proliferation, differentiation and apoptosis though distinct mechanisms [70,84–86], and the negative and positive association of their expression levels, respectively, with breast cancer risk and adiposity [87–89]. *In vitro*, the isoflavone GEN has been shown to enhance secretion (hence, availability as endocrine/paracrine signals) of adiponectin [27] and to inhibit that of leptin [90]. The bioactive component chitosan from edible mushrooms, which was found to demonstrate antiobesogenic activity in rats [91], similarly reduced visceral adipose tissue leptin levels in mice consuming chitosan-supplemented diet [92]. Further, the short-chain fatty acid propionic acid, which is produced by the colonic fermentation of dietary fiber known to be preventive for the development of obesity [93], was shown to increase leptin messenger RNA expression and corresponding protein secretion, in the absence of coincident effects on adiponectin, in human omental and subcutaneous adipose tissue explants [94]. While the increased secretion of leptin by propionic acid appears counterintuitive to its antiobesity and, by extension, anticipated antimammary tumorigenic effects, this was accompanied by the reduced expression of the proinflammatory factor adipokine resistin, suggesting that the repertoire of adipokines presented to target cells may predict the final growth/proliferative outcome. In this regard, a recent study has shown significantly elevated plasma resistin levels in patients with breast cancer relative to those without disease [95], consistent with the link between inflammation and breast cancer risk.

Our group's approach to mechanistically address the directional signaling from stromal to epithelial cells initiated by bioactive dietary factor targeting of mammary fat pad involves (1) defining the *in vivo* measures of mammary epithelial and stromal differentiation upon early dietary SPI exposure and (2) recapitulating these responses in nontumorigenic mammary epithelial cells exposed to conditioned medium from differentiated 3T3-L1 adipocyte treated with GEN *in vitro* [27]. While our experiments constitute proof of concept, there are caveats that require further scrutiny. Our studies did not unequivocally identify GEN-specific gene targets in stromal fibroblasts and adipocytes distinct from those of epithelial cells, since the gene expression analyses were carried out using whole mammary tissues. Moreover, the biological and molecular outcomes observed *in vitro* with GEN precluded the contribution of other SPI-associated bioactive components, which may elicit more direct effects than could be attributed to GEN alone. Finally, it was not possible to demonstrate the converse directional signaling (i.e., from epithelial to stromal compartment) that may equally underlie mammary tumor prevention. In support of the existence of epithelial-to-stromal dialog, it was shown that during the development of breast cancer, the stromal compartment responded to signals from tumorigenic cells, leading to a more 'reactive' stroma and amplification of the tumorigenic state [96]. Additional studies using isolated adipocytes and fibroblastic cells derived from mammary fat pad or *in vivo* sampling of mammary fat pad followed by proteomic analyses [65,97], as a function of whole diets and purified bioactive components, will provide a 'glimpse' of the mammary secretome and presumably regulators of mammary stromal-mediated epithelial changes.

The elegant study by Lam et al. [70] demonstrating the precise role of adiponectin in mammary carcinogenesis can serve as a paradigm for mechanistically elucidating the role of adipocyte-specific gene targets of diet and dietary factors on mammary tumor prevention. In that study, MMTV-polyomavirus middle T-antigen transgenic mice with reduced adiponectin expression were generated to test the effects of adiponectin haploinsufficiency on the promotion of mammary tumors. Similar kinds of studies could be performed to test the function of candidate mammary adipocyte genes that are identified from gene expression analyses of tissues from rodent models under different dietary programs. In this regard, the recent report on the characterization of a 5.4-kb adiponectin promoter/5′ regulatory region that confers adipocyte-specific expression of target genes may provide an avenue for studying gene function in the context of bidirectional signaling in the mammary gland [98]. While it is unknown whether mammary adipose tissue exhibits specialized responses to extracellular signals or displays gene expression patterns distinct from retroperitoneal (subcutaneous) adipose tissue, an earlier study showed that the lipid composition in adipose tissue of virgin rat mammary glands resemble that of the retroperitoneal adipose [99].

5. Concluding remarks

The notion that the mammary fat pad is a direct target of bioactive dietary factors for mammary tumor protection is not difficult to envision, given that in any biological system, nothing stands alone. It is perhaps paradoxical that studies to address this remain relatively limited and the concept that bidirectional signaling within the mammary microenvironment for breast cancer prevention remains an intriguing observation. While the stromal compartment is not the main target of carcinogens [100], the possibility that a very early event upon carcinogenic insult is the sensing by stromal cells of 'something amiss' in adjacent epithelial cells is not unlikely. If this is the case, the identification of mammary fibroblast- and adipocyte-specific 'early' molecular targets by bioactive components in model systems may eventually provide biomarkers for the very early stages of the disease. The recent characterization of a mammary stromal fibroblastic cell line from mice that can differentiate to a preadipocyte lineage [101] in coculture studies with nontumorigenic or tumorigenic mammary epithelial cells will enable a proof-of-principle evaluation of the epithelial/stromal adipocyte dialog and associated mediators.

The findings that mammary stroma can reprogram testicular and neural stem cells to produce progeny committed to a mammary epithelial cell fate [102,103] and that a precancerous mammary stem cell may be programmed to become breast cancer [104] suggest the possibility that direct dietary factor effects on mammary stroma may alter stem cell behavior to inhibit neoplastic transformation. Thus, while mammary stem cells may constitute direct targets of bioactive dietary components as recently suggested by the report that curcumin added *in vitro* can induce mammosphere-forming ability in normal and malignant breast cells [105], a dual effect of dietary factors on mesenchymal and epithelial stem cells is also likely.

Further, dietary factors may directly influence the stem cell compartment in mammary stroma at the levels of the preadipocyte pool and the number of multipotent stem cells that enter the adipocyte lineage. The effects of obesity, high fat diets and other dietary factors on mammary preadipocyte populations remain unknown. It has been suggested that the inability of a particular adipose depot to expand may be causative in the accumulation of hypertrophic adipocytes and a predisposing factor in metabolic disease. Hence, it is possible that certain diets or dietary factors may mediate indirect beneficial actions on mammary epithelial cells via their modulation of preadipocyte commitment and/or differentiation of new mammary adipocytes. A recent report that *in utero* exposure to the environmental agent tributylin induced multipotent stem cells to differentiate into adipocytes provides strong support to this possibility [106].

Finally, while the contribution of inflammatory/immune cells found in mammary stroma is not included in the present review, their relevance as dietary factor targets to mediate epithelial proliferation and differentiation cannot be ignored, given that local inflammation associated with solid tumors is partly a consequence of immune cells in the tumor stroma [107]. Indeed, we observed that immune-related genes constitute major targets of dietary exposure to SPI and GEN in mammary epithelial cells of young adult rats [50]. The down-regulated expression of epithelial genes involved in antigen presentation, antigen processing and inflammation, including that of interleukin 17β, a homolog of interleukin 17, which is linked to neutrophil chemotaxis, suggests the possibility of similar specific targeting of immune cells localized

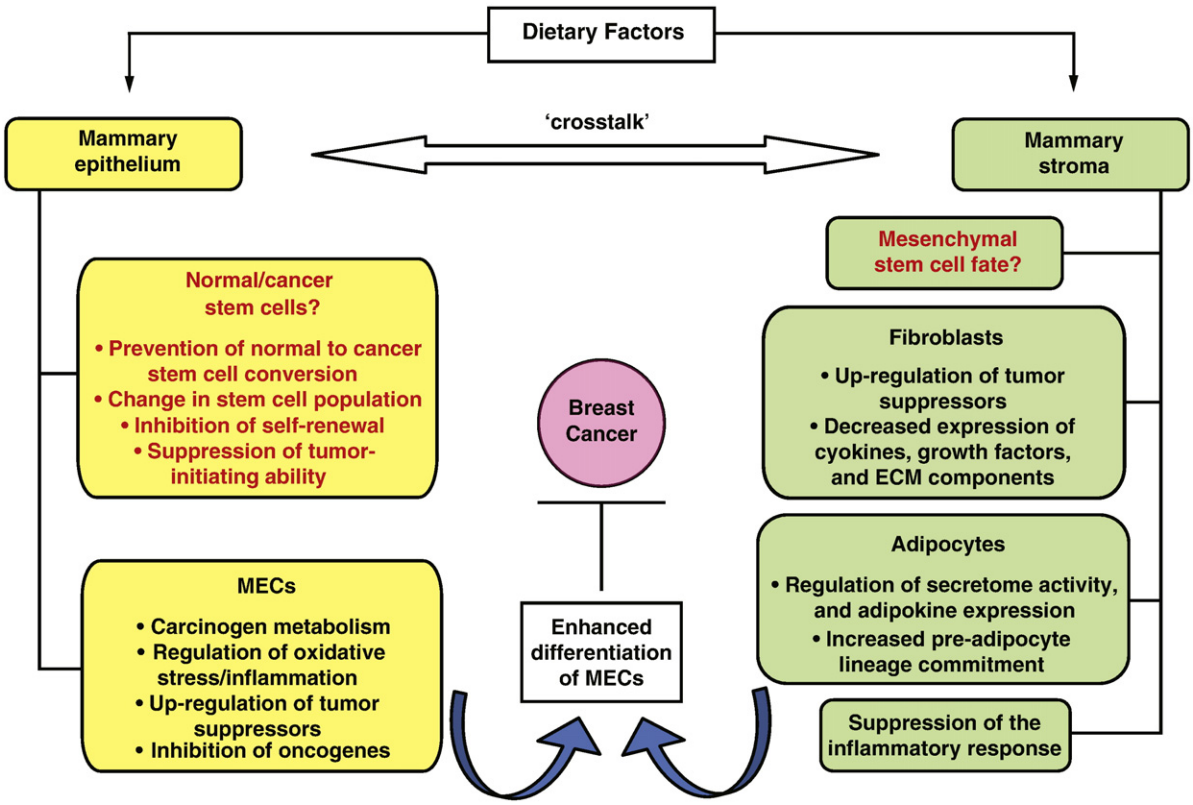

Fig. 2. A proposed model of cellular processes regulated by dietary factors in mammary epithelial and stromal compartments for breast cancer protection. The bidirectional arrows indicate an ongoing dialog between the mammary compartments. Mammary epithelial and mesenchymal stem cells are considered to represent cells of origin for each compartment. The composite actions of each mammary cell type result in the enhanced differentiation and, hence, increased resistance of mammary epithelial cells to carcinogenic insults, leading to decreased breast cancer risk.

to stroma and is consistent with promotion by the immune microenvironment of tumor progression [107].

In summary, bidirectional signaling between mammary stroma and epithelial cells promoted by bioactive dietary components constitutes a relevant biological event for mammary tumor prevention (Fig. 2). Thus, it is essential that, in future studies where dietary factor effects are described for mammary tumor prevention, their contributions to the phenotype and molecular profiles of mammary stromal fibroblasts and adipocytes are investigated coincident with those of neighboring epithelium. Gaining a better understanding of the complex interrelationships among the different mammary compartments in response to environmental ('dietary') cues may expand nutritional strategies for breast cancer prevention and therapeutic interventions.

Acknowledgments

We thank Dr. Frank A. Simmen (UAMS) for critical comments on the manuscript. Work described from Dr. R.C.M. Simmen's laboratory was supported in part, by grants from the USDA-CRIS 6251-5100002-06S, Department of Defense Breast Cancer Research Program (CDMRP W81XWH-08-0548), the Fashion Footwear Association of New York (FFANY/QVC) and the Arkansas Children's Hospital Research Institute/Children's University Medical Group Program.

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Paracrine-Acting Adiponectin Promotes Mammary Epithelial Differentiation and Synergizes with Genistein to Enhance Transcriptional Response to Estrogen Receptor β Signaling

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Mammary stromal adipocytes constitute an active site for the synthesis of the adipokine, adiponectin (APN) that may influence the mammary epithelial microenvironment. The relationship between "local," mammary tissue-derived APN and breast cancer risk is poorly understood. Here, we identify a novel mechanism of APN-mediated signaling that influences mammary epithelial cell proliferation, differentiation, and apoptosis to modify breast cancer risk. We demonstrate that early dietary exposure to soy protein isolate induced mammary tissue APN production without corresponding effects on systemic APN levels. In estrogen receptor (ER)-negative MCF-10A cells, recombinant APN promoted lobuloalveolar differentiation by inhibiting oncogenic signal transducer and activator of transcription 3 activity. In ER-positive HC11 cells, recombinant APN increased ERβ expression, inhibited cell proliferation, and induced apoptosis. Using the estrogen-responsive 4X-estrogen response element promoter-reporter construct to assess ER transactivation and small interfering RNA targeting of ERα and ERβ, we show that APN synergized with the soy phytoestrogen genistein to promote ERβ signaling in the presence of estrogen (17β-estradiol) and ERβ-specific agonist 2,3-*bis*(4-hydroxyphenyl)-propionitrile and to oppose ERα signaling in the presence of the ERα-specific agonist 4,4′,4′-(4-propyl-(1*H*)-pyrazole-1,3,5-triyl)trisphenol. The enhancement of ERβ signaling with APN + genistein cotreatments was associated with induction of apoptosis, increased expression of proapoptotic/prodifferentiation genes (*Bad*, *p53*, and *Pten*), and decreased antiapoptotic (*Bcl2* and *survivin*) transcript levels. Our results suggest that mammary-derived APN can influence adjacent epithelial function by ER-dependent and ER-independent mechanisms that are consistent with reduction of breast cancer risk and suggest local APN induction by dietary factors as a targeted approach for promotion of breast health. **(*Endocrinology* 152: 3409–3421, 2011)**

Obesity (assessed by body mass index) increases the risk of breast cancer in postmenopausal women by 30–50% (1–3), in part due to aromatization of androstenedione to estrone by adipose tissue and subsequent conversion to the active hormone estradiol. Further, obese women at diagnosis are more likely to have higher grade and poor prognosis regardless of their menopausal status (4–6). The adipose tissue produces and secretes at least 50 different polypeptide hormones termed adipokines that can act in an endocrine, paracrine, or autocrine manner,

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2011-1085 Received April 6, 2011. Accepted June 7, 2011.
First Published Online June 28, 2011

Abbreviations: APN, Adiponectin; APNR, APN receptor; CAS, casein; CSS, charcoal-stripped serum; DPN, 2,3-*bis*(4-hydroxyphenyl)-propionitrile; E_2, 17β-estradiol; EGF, epidermal growth factor; ER, estrogen receptor; ERE, estrogen response element; 4xERE-TK-Luc, 4xERE-TK-Luciferase; GEN, genistein; MEC, mammary epithelial cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PND, postnatal day; PPT, 4,4′,4″-(4-propyl-(1*H*)-pyrazole-1,3,5-triyl)trisphenol; PTEN, phosphatase and tensin homolog deleted on chromosome ten; QPCR, quantitative real-time PCR; scRNA, scrambled RNA; siRNA, small interfering RNA; SPI, soy protein isolate; STAT3, signal transducer and activator of transcription 3; TK, thymidine kinase.

thus changing the notion of this tissue from a simple fat depot into a very active endocrine organ (7, 8). Unlike most adipokines, serum adiponectin (APN) level is lower in obese individuals compared with normal weight or lean subjects (9) and is considered to be a link between obesity and breast cancer (10–14).

APN effects are mediated through two types of receptors: APN receptor (APNR)1, which is expressed ubiquitously and has higher affinity for the low molecular weight APN trimer, and APNR2, expressed mainly in the liver and has similar affinity for the low and high molecular weight forms of APN (15). Physiological doses of APN inhibit cell proliferation and/or induce apoptosis of both estrogen receptor (ER)-negative (MDA-MD231) and positive (T47D and MCF-7) breast cancer cell lines in a cell type-specific manner (16–18). A recent study using mouse mammary tumor virus-polyomavirus middle T antigen transgenic mice with decreased APN expression demonstrated that *in vivo* APN haploinsufficiency facilitates mammary tumorigenesis by down-regulation of tumor suppressor phosphatase and tensin homolog deleted on chromosome ten (PTEN) activity and activation of phosphatidylinositol 3 kinase/AKT signaling (19).

A role for estrogen in the etiology of breast cancer is supported by increased risk of the disease during conditions of prolonged estrogen exposure, such as early menarche, late menopause, late first full-term pregnancy, and nulliparity (20). Biological effects of estrogen are mediated mainly by two members of the nuclear receptor superfamily, ERα and ERβ. These ER isoforms can form homo- or heterodimers and, via the classical pathway, bind to estrogen response elements (ERE) in target genes or, through nonclassical pathways, interact with other transcription factors (21). Both receptors are coexpressed in approximately 70% of breast tumors, and although ERα is associated with cell proliferation and ERβ with antiproliferative effects, the exact role of ERβ in breast cancer remains controversial (22–24). However, ERα/ERβ ratio is higher in breast tumors compared with normal tissue due to loss of ERβ expression during tumor progression (25–27), suggesting a tumor suppressor role for ERβ (28–33). Genomic and proteomic expression analysis of breast cancer cells indicate that when both ER are coexpressed, ERβ inhibits the overall proliferative/survival actions of ERα (34–43).

The soy isoflavone genistein (GEN) is considered to partly mediate the protective effects of soy-rich diets against breast cancer (44, 45) by its preferential activation of ERβ signaling, as shown by enhanced recruitment of steroid receptor coactivator more strongly to ERβ in the presence of GEN (40, 46). In breast cancer cells expressing both ER, GEN inhibits the proliferative actions of ERα by increasing the expression of a number of ERβ-mediated proteins involved in apoptosis, cell cycle, motility, and lipid metabolism (47). Further, APN induces the expression of both ER in malignant mammary epithelial cells (MEC) (48), suggesting that APN may exert its antitumor effects by regulating the direction of ERα and/or ERβ signaling. However, whether APN similarly functions in normal (nontumorigenic) MEC to influence ERα/ERβ cross talk and, more importantly, whether this function of APN is coregulated by GEN have not been determined.

Here, we evaluated the hypothesis that dietary induction of APN synthesis and/or secretion by mammary stromal adipocytes leads to enhancement of ERβ signaling on neighboring MEC by paracrine-acting APN. We show that lifetime dietary exposure of weaning and young adult female rats to soy protein isolate (SPI) increased APN protein levels in mammary tissue without parallel effects on systemic APN levels. Using nonmalignant human (MCF-10A) and mouse (HC11) MEC, we demonstrate that recombinant APN can enhance differentiation of ER-negative MCF-10A cells by its suppression of basal signal transducer and activator of transcription 3 (STAT3) activity and promote apoptosis and differentiation of ER-positive HC11 cells by its activation of ERβ signaling in cooperation with the ERβ-specific ligand GEN. Further, using specific agonists to ERα [4,4′,4″-(4-propyl-(1*H*)-pyrazole-1,3,5-triyl)trisphenol (PPT)] and ERβ [2,3-*bis*(4-hydroxyphenyl)-propionitrile (DPN)] along with small interfering RNA (siRNA) technologies targeting either receptor isoform, we define novel synergistic roles for APN and GEN in promoting ERα/ERβ cross talk in MEC.

Materials and Methods

Animals and diets

All animal experiments were carried out under protocols approved by The University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee. Time-mated Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were individually housed in polycarbonate cages under conditions of 24 C, 40% humidity, and a 12-h light, 12-h dark cycle. At gestation d 4, dams were randomly assigned to one of two semipurified isocaloric diets containing either casein (CAS) (New Zealand Milk Products, Santa Rosa, CA) or SPI (Solae, St. Louis, MO) as sole protein source and formulated following the American Institute of Nutrition-93G guidelines (49), except that corn oil was substituted for soybean oil. SPI contains the isoflavones GEN (216 ± 2 mg/kg) and daidzein (160 ± 6 mg/kg) as aglycone equivalents. Animals were provided food and water *ad libitum*. At delivery, all pups from dams of the same diet groups were pooled, and 10 pups (five per sex) were randomly assigned to each dam to nurse. Female pups were weaned at postnatal day (PND)21 to the same diets as their dams and were fed this diet throughout the study. The inguinal mammary glands (number 4)

were collected at PND21 and at PND50 (n = 5 female offspring per PND) and processed for Western blot analyses as described below.

Cell culture and treatments

The mouse MEC line HC11 (kindly provided by Jeffrey M. Rosen (Baylor College of Medicine, Houston, TX) and the human nontumorigenic MEC line MCF-10A (American Type Culture Collection, Manassas, VA) were maintained in growth medium at 37 C in a 5% CO_2 incubator as previously described (50, 51). Phenol red-free media supplemented with charcoal-stripped serum (CSS) was used for serum starvation (0.5% CSS) and treatments (2.5% CSS). Recombinant mouse or human APN (R&D Systems, Inc., Minneapolis, MN) dissolved in PBS was used at 8 μg/ml and GEN (Sigma Chemical Co., St. Louis, MO) dissolved in dimethylsulfoxide was used at 40 nM. Treatments with PBS and dimethylsulfoxide served as negative controls.

Serum APN levels

The concentrations of APN in sera of CAS- and SPI-fed rats collected at PND21 and PND50 were measured using a rat APN ELISA kit (Linco Research, St. Charles, MO). The assay sensitivity was 0.15 ng/ml, and intra- and interassay variations were less than 8.5%.

Antibodies and Western blot analysis

Whole-cell extracts were prepared and immunoblotted following previously described protocols (50, 52). Antimouse APN (Abcam, Inc., Cambridge, MA), antiphospho-STAT3 Tyr705 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-STAT3 (Santa Cruz Biotechnology, Inc.) antibodies were each used at 1:1000 dilution. Anti-α-tubulin (Santa Cruz Biotechnology, Inc.) and anti-β-actin (Sigma Chemical Co.) antibodies at 1:2000 dilutions were used as normalizing controls for protein loading. Blots were stripped with Restore Western blot stripping buffer (Pierce Biotechnology, Rockford, IL) before reprobing with additional antibodies. Immunoreactive signals were visualized using Amersham ECL Plus (GE Healthcare Life Sciences, Piscataway, NJ) and quantified using the Bio-Rad molecular analyst detection system and Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Quantitative real-time PCR (QPCR)

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed to cDNA using iScript cDNA synthesis kit (Bio-Rad Laboratories). QPCR was carried out using the SYBR Green Supermix (Bio-Rad Laboratories) and ABI Prism 7000 Detection System (Applied Biosystems, Foster City, CA). Primers (Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org) for PCR were designed to span introns using Primer Express software (Applied Biosystems) and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The expression of each target mRNA was calibrated to a standard curve generated using pooled cDNA stocks and normalized to that of TATA-box binding protein (*Tbp*).

Cell viability and numbers

The number of viable cells was evaluated using a cell proliferation assay kit [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); American Type Culture Collection] according to the manufacturer's instructions. Cells (20,000 cells/well) were seeded in 96-well plates and treated with APN (8 μg/ml) or vehicle (PBS) every 2 d for 6 d. Absorbance values (570 nm) reflect the ability of metabolically active cells to reduce the yellow tetrazolium MTT salts into a purple precipitate. Viable cell numbers were determined under the same treatment conditions by Trypan blue exclusion method (INC Biomedicals, Inc., Aurora, OH). Each experiment was conducted in quadruplicate and repeated twice.

Acini morphogenesis assay

MCF-10A cells were seeded on a layer of Matrigel (BD Biosciences, San Jose, CA) in eight-well chamber slides and allowed to form acini as previously described (52). Culture medium containing 2% charcoal-stripped horse serum and 5 ng/ml epidermal growth factor (EGF) without (vehicle alone) or with added APN (8 μg/ml) was refreshed every 4 d. At least 80 acini were counted from five random areas per chamber ($\times$20 objective), with four chambers for each treatment group. Acini number and diameter were assessed at d 12 of culture using a phase contrast microscope (Carl Zeiss AG, Oberkochen, Germany) ($\times$20 objective). Confocal images of 4′,6-diamidino-2-phenylindole-stained acini were collected on a Zeiss LSM510 confocal microscope ($\times$20 objective).

Fluorescence-activated cell sorting

Untreated and treated HC11 cells were harvested with trypsin, washed with ice-cold PBS, and fixed with ice-cold 70% ethanol. After propidium iodide (10 μg/ml) staining, at least 10,000 cells were analyzed using a Becton Dickinson FACSCalibur (BD Biosciences). Histograms were generated with the CellQuest software program (BD Biosciences).

Transient transfection and luciferase assays

The 4xERE-thymidine kinase (TK)-Luciferase reporter construct was generously provided by Benita S. Katzenellenbogen (University of Illinois, Urbana-Champaign, IL). HC11 cells were cotransfected with the reporter plasmid or empty vector (pGL3B) (each added at 0.5 μg/well) and with *Renilla*-Luciferase construct (50 ng/well) using Lipofectamine 2000 (Invitrogen), as previously described (52, 53). Incubation with 17β-estradiol (E_2) (10 nM), DPN (40 nM), PPT (40 nM), or GEN (40 nM) was carried out without or with APN (8 μg/ml) pretreatment for 24 h. Cells were lysed in lysis buffer (Promega, Madison, WI) and quantitative determination of luciferase activity used a Dual-Luciferase Reporter Assay System (Promega) and a MLX Microplate Luminometer (Dynex Technologies, Chantilly, VA). ERE-Luc activity was normalized to that of *Renilla* luciferase, which served as an internal control for transfection efficiency. Data are presented as means $\pm$ SEM from at least three independent experiments, with each experiment performed in quadruplicates.

Apoptosis assay

HC11 cells were seeded in white-walled 96-well plates (15,000 cells/well) and treated with E_2 (10 nM) in the presence or absence of GEN (40 nM) and/or APN (8 μg/ml) for 72 h. Cell apoptosis was determined by quantifying caspase-3 and caspase-7 activity using the luminometric Caspase-Glo 3/7 assay

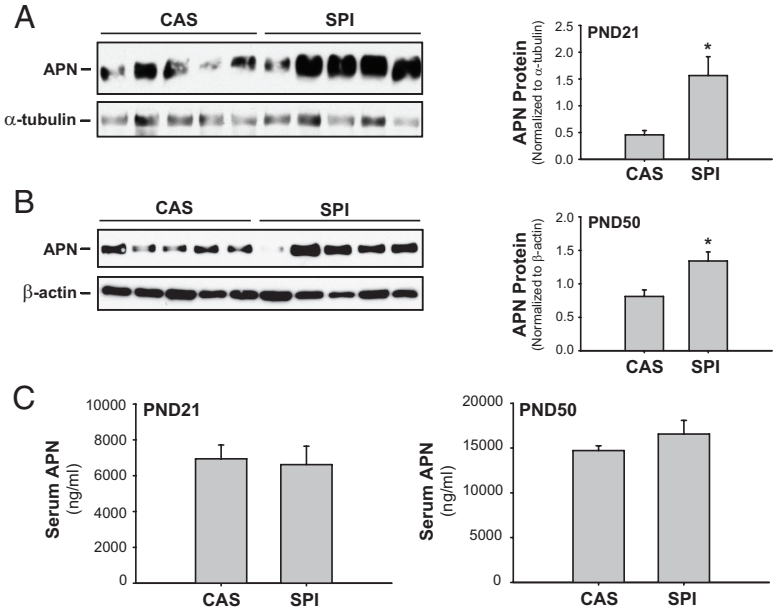

FIG. 1. Dietary SPI induces local APN protein expression in mammary tissue. A and B, *left*, Western blot analysis of APN protein in mammary tissue of female rat offspring at PND21 (A) or PND50 (B) exposed to CAS or SPI diets. *Each lane* represents an individual animal and contains 50 μg of total protein. *Right*, Immunoreactive bands were quantified by densitometry and values normalized to those of loading control α-tubulin or β-actin, respectively, and are presented as histograms; *, $P < 0.05$ relative to CAS. C, Serum APN levels were quantified in weanling (PND21) or young adult (PND50) rats using a rat APN ELISA kit as described in *Materials and Methods* (n = 7 rats per diet, per PND).

kit (Promega) following the manufacturer's protocol and a MLX Microplate Luminometer (Dynex Technologies).

Data analysis

Computer-assisted statistical analyses were performed using the StatView 5.0 program for Windows. Data were analyzed by Student's *t* test, one-way ANOVA, or two-way ANOVA. Differences between means in two-way ANOVA were further analyzed by Tukey's test. A value of $P < 0.05$ was considered significant.

Results

Dietary SPI increases mammary APN expression

To date, most studies linking APN to obesity-related breast cancer are based on systemic APN levels; hence, the relationship between mammary APN expression and breast cancer occurrence has not been fully determined. Here, we examined whether early exposure to dietary SPI that promoted mammary epithelial differentiation *in vivo* (54) and conferred protection from chemical-induced mammary tumor formation in female rat offspring (55, 56) is associated with higher APN production in mammary adipocytes. Mammary tissues from female rats exposed to dietary SPI or the control diet CAS beginning at gestation d 4 until tissue collection at PND21 and at PND50 were assessed for APN expression by Western blotting. Levels of APN protein (molecular mass 30 kDa) were higher in mammary tissues of weanling and young adult rats exposed to SPI than to CAS diets (Fig. 1, A and B). Serum APN levels increased with age (PND50 > PND21) (Fig. 1C) and were within the reported physiological range for humans and mice (57). Despite the lower body weights of PND21 and PND50 rats exposed to dietary SPI when compared with the control group (54), there was no comparable increase in systemic APN levels with diet, as was shown for mammary tissue (Fig. 1, A–C). These data provide the first *in vivo* evidence for dietary regulation of "local" mammary APN expression and suggest that mammary adipose-derived APN may be a mediator of the mammary tumor protective effects of dietary SPI.

APN inhibits mammary epithelial proliferation and promotes cellular apoptosis

The tumor protective actions of APN on breast epithelium are likely mediated at multiple levels, including proliferation, apoptosis, insulin sensitivity, growth factor sequestration, recruitment of proinflammatory cytokines, and angiogenesis (58). To mechanistically dissect the functional outcomes of greater mammary adipocyte APN production/secretion with SPI dietary exposure (Fig. 1, A and B) on neighboring epithelial cells, mouse nontumorigenic MEC HC11 were treated with recombinant mouse APN (8 μg/ml) and assessed for cell proliferation/viability and apoptosis status relative to control (vehicle only treated) cells. Cells administered growth media without added EGF for the same period as APN (no EGF, Fig. 2A) served as positive control in these experiments. APN treatment for 24 or 48 h decreased cell viability, as measured by the MTT assay, relative to control cells (Fig. 2A). Consistent with the MTT assay, APN treatment decreased cell numbers ($0.64 \pm 0.03 \times 10^6$) relative to control group ($0.83 \pm 0.02 \times 10^6$; $P < 0.05$) (Fig. 2B). Fluorescence-activated cell sorting analysis of cells treated with APN for 12 h showed an increase in the percentage of cells in the sub-G_0 (apoptotic) phase, with no changes noted at other cell cycle stages (Fig. 2, C and D). The higher apoptotic status with APN treatment was correlated with an early, although transient, decrease (compare 6 and 24 h) in transcript levels for survivin (Fig. 2E), the antiapoptotic protein normally up-regulated in human breast cancer (59), relative to untreated cells. APN did not alter the expression

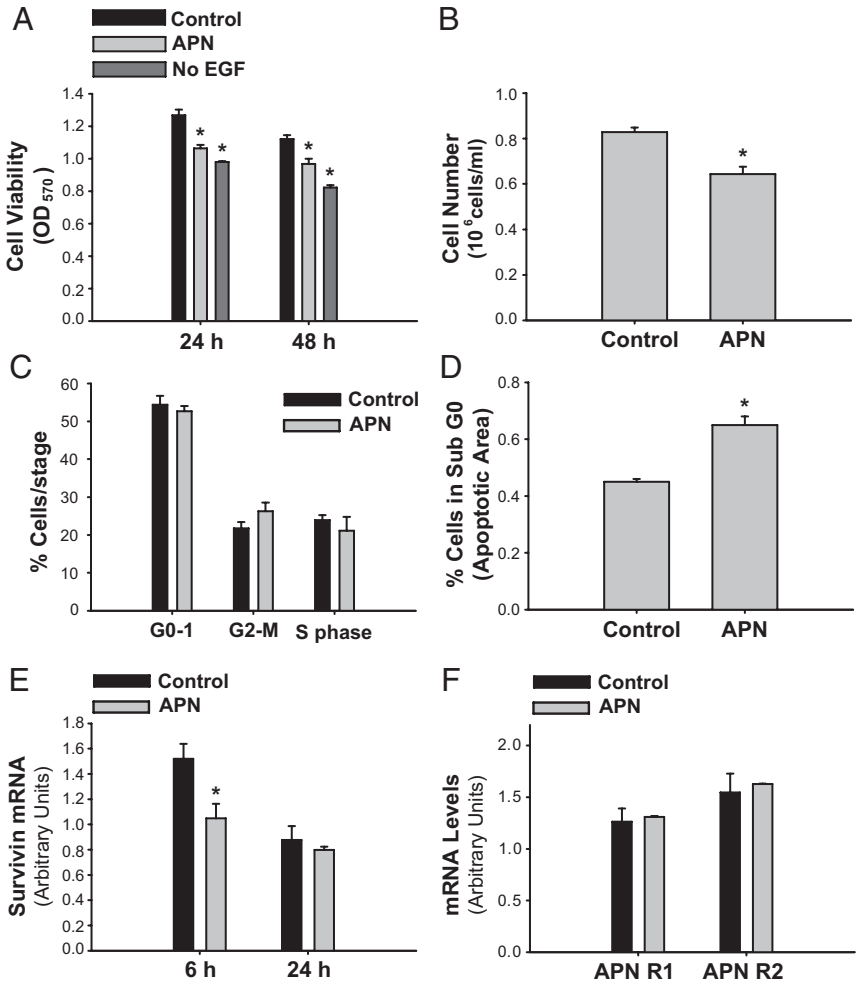

FIG. 2. APN inhibits proliferation and promotes apoptosis of ER-positive HC11 cells. A, HC11 cells treated with APN (8 μg/ml) for 24 or 48 had decreased cell proliferation compared with control cells (vehicle treated) as measured by the MTT assay. Cells incubated in medium without added EGF for the same duration served as a positive control for decreased cell growth. B, Cells were seeded in 100-mm dishes and treated in same manner as for MTT assay. Viable cells were quantified by the trypan blue exclusion method after 12 h of treatment using a hemocytometer. C and D, Control and APN-treated cells were analyzed by fluorescence-activated cell sorting. E and F, Transcript levels of the antiapoptotic protein survivin and of APNR1 and APNR2 were quantified by QPCR and normalized to *Tbp*. Results are mean ± SEM from two independent experiments performed in triplicate; *, $P < 0.05$ relative to control.

of its receptors APNR1 and APNR2 (Fig. 2F), which have been shown to mediate its pleiotropic actions in target cells (15).

APN enhances mammary epithelial differentiation

To further address APN regulation of mammary epithelial function as an underlying mechanism for mammary tumor protection, APN effects on differentiation were assessed in two MEC lines using distinct outcomes. Expression of β-casein mRNA levels, a marker of mammary epithelial differentiation, was evaluated in the ER-positive HC11 cells treated with recombinant mouse APN or vehicle, in the presence of ovine prolactin (5 μg/ml; ovine prolactin-21, AFP-10692C) for 48 h. As shown in Fig. 3A, APN up-regulated β-casein mRNA levels by 6.2-fold ($P = 0.006$) relative to control (vehicle) cells. In the ER-negative, nontumorigenic human MEC MCF-10A plated on Matrigel-coated chamber slides, APN promoted the formation of acini structures with hollow lumen (Fig. 3, B and C), resembling the morphogenesis of the mammary gland during pregnancy (60). The shift toward the formation of larger acini [40–90 ($\times 10^2$) μm^2 range] from the smaller-sized structures [<10 ($\times 10^2$) μm^2 range] was observed as early as d 6 (data not shown) and persisted through d 12 of APN treatment (Fig. 3, B–D), indicating induction by APN of early lobuloalveolar differentiation. These results suggest that APN promotes the differentiation of nontumor MEC, irrespective of ER status.

In a previous study using genome-wide profiling of mammary tissue of weanling rats exposed to CAS or SPI via maternal diet (54), we found that expression levels of the transcription factor STAT3 were attenuated with dietary exposure to SPI, relative to CAS. Given the reported inhibition by APN of STAT3 signaling (61), the constitutive activation (measured as tyrosine phosphorylation) of STAT3 in breast cancer (62), and findings that promotion of tumor cell survival is partly mediated by activated STAT3 through up-regulation of the antiapoptotic protein survivin (59, 62), we evaluated whether enhanced differentiation of MEC HC11 and MCF-10A induced by APN is mediated by APN inhibition of STAT3 signaling. Western blot analyses showed comparable levels of total STAT3 protein (using anti-STAT3 antibodies) in control and APN-treated HC11 and MCF-10A cells (Fig. 3, E and F). However, whereas HC11 cells demonstrated undetectable/low activated STAT3 levels (anti-pSTAT3 immunoreactivity) with or without APN treatment (Fig. 3E), MCF-10A cells showed robust STAT3 activity that was significantly attenuated by APN (Fig. 3F). These results suggest that context-dependent signaling mechanisms may underlie the biological response of MEC to the protective effects (*e.g.* increased differentiation) of APN.

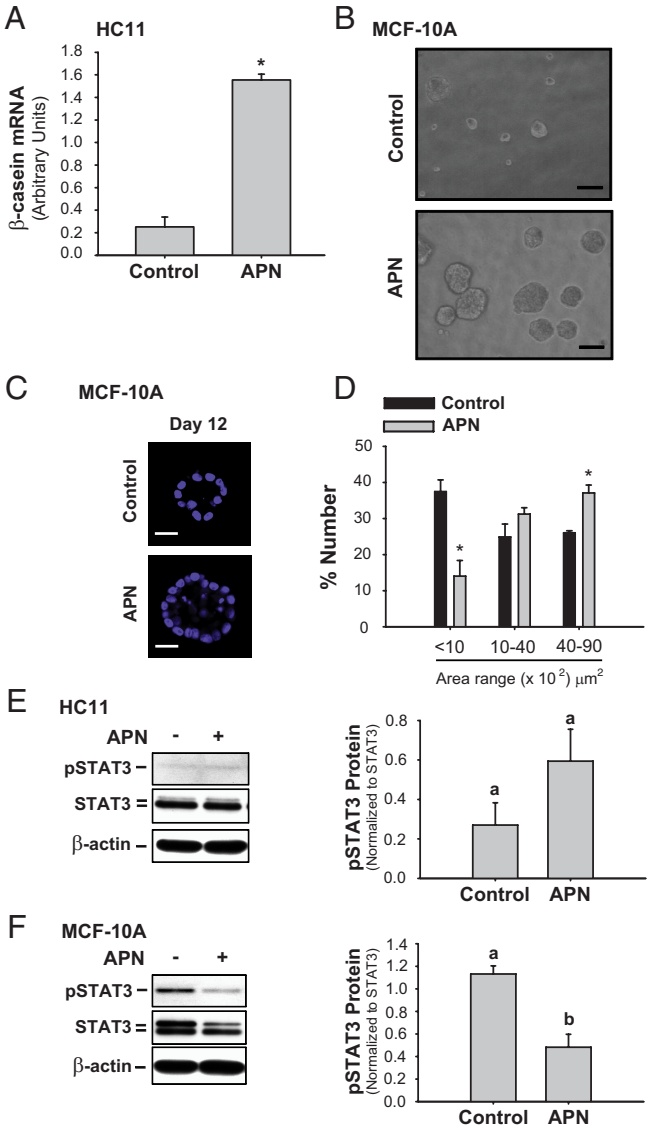

FIG. 3. APN enhances differentiation of MEC. A, Gene expression of the mammary gland differentiation marker β-CAS was quantified in HC11 cells exposed to prolactin for 48 h and then treated with APN (8 μg/ml) or vehicle for 24 h. B–D, MCF-10A cells were plated on a layer of Matrigel and allowed to form acini with hollow lumens as described in *Materials and Methods*. B, Phase contrast images of acini treated with vehicle (control) or APN. *Scale bar*, 50 μm. C, Z-sections of 4′,6-diamidino-2-phenylindole-stained acini showing hollow lumen and morphology at d 12 of culture were recorded by confocal imaging. *Scale bar*, 20 μm. D, The numbers of acini were counted at d 12 of mammary acini morphogenesis from five randomly chosen areas per chamber with four chambers per treatment group. Results are mean ± SEM from two independent experiments; *, $P < 0.05$ relative to control. E and F, HC11 or MCF-10A cells were treated with mouse or human recombinant APN for 18 h, respectively, and whole-cell extracts were prepared. Protein levels of phosphorylated STAT3 (pSTAT3) (*upper panel*), total STAT3 (*middle panel*), and β-actin (*lower panel*) were analyzed in cell lysates (20 μg of total protein) by Western blotting. Immunoreactive bands were quantified by densitometric scanning. Values normalized to those of loading control β-actin are presented as histograms (*right panel*). Representative blots from three independent experiments with similar results are shown; *, $P < 0.05$ relative to control.

APN promotes ERβ transcriptional activity

In light of earlier findings suggesting an inverse estrogen (E_2)/APN connection in human breast cancer cells (63), we addressed the participation of ER on APN signaling in the nonmalignant, ER-positive HC11 cells. We first evaluated whether APN altered the expression levels of ERα and ERβ isoforms in these cells at the mRNA and protein levels, by QPCR and Western immunoblottings, respectively. Cells treated with APN for 6 h showed lower ERα (2-fold) and higher ERβ (2-fold) transcript levels, relative to control (vehicle treated) cells (Fig. 4A). A more robust effect (~10-fold) of APN on ERβ protein expression was observed when compared with vehicle-treated cells, in contrast to the lack of effect noted on ERα protein levels (Fig. 4, B and C). On a per protein basis, however, expression levels of ERα were greater than those of ERβ in these cells. Thus, APN may promote ERβ signaling in HC11 cells by increasing ERβ relative to ERα gene and protein expression.

To evaluate the functional consequence of the preferential induction by APN of ERβ relative to ERα expression in mammary epithelium, the transcriptional response of HC11 cells to ERβ agonists DPN and GEN (20-fold higher affinity for ERβ than for ERα) (40) were compared with those of E_2 (binds ERα and ERβ with similar affinities) and ERα-specific agonist PPT, in control and APN-treated cells. Cells were transfected with the estrogen-responsive 4xERE-TK-Luciferase (4xERE-TK-Luc) construct as a reporter for ER transactivation. In cells transfected with the 4xERE-TK-Luc plasmid, treatment with E_2 and with PPT resulted in similar (2-fold) increases in Luc promoter activity, relative to untreated cells (Fig. 4D). GEN elicited a modest, although significant, increase in Luc reporter activity relative to nontreated cells; the magnitude of the increase was lower than that obtained with E_2 and PPT treatments (Fig. 4D). DPN-treated cells displayed Luc reporter activity that, although not statistically significant, was numerically higher to those of untreated cells. Because both ligand-activated ER isoforms are known to bind the ER recognition sequence in the 4xERE-TK-Luc construct with comparable affinities, results are consistent with the predominant expression of ERα relative to ERβ in non-APN-treated HC11 cells (Fig. 4, B and C). Interestingly, APN treatment had no effect on the magnitude of ER-mediated transcriptional responses individually elicited by E_2, DPN, or GEN (which all bind ERβ) (Fig. 4D), despite APN induction of ERβ expression levels in these cells (Fig. 4, A–C). These collective data suggest that APN actions may be manifest only under specific cellular contexts, possibly when both ER are functionally active (*i.e.* ligand activated) or under conditions

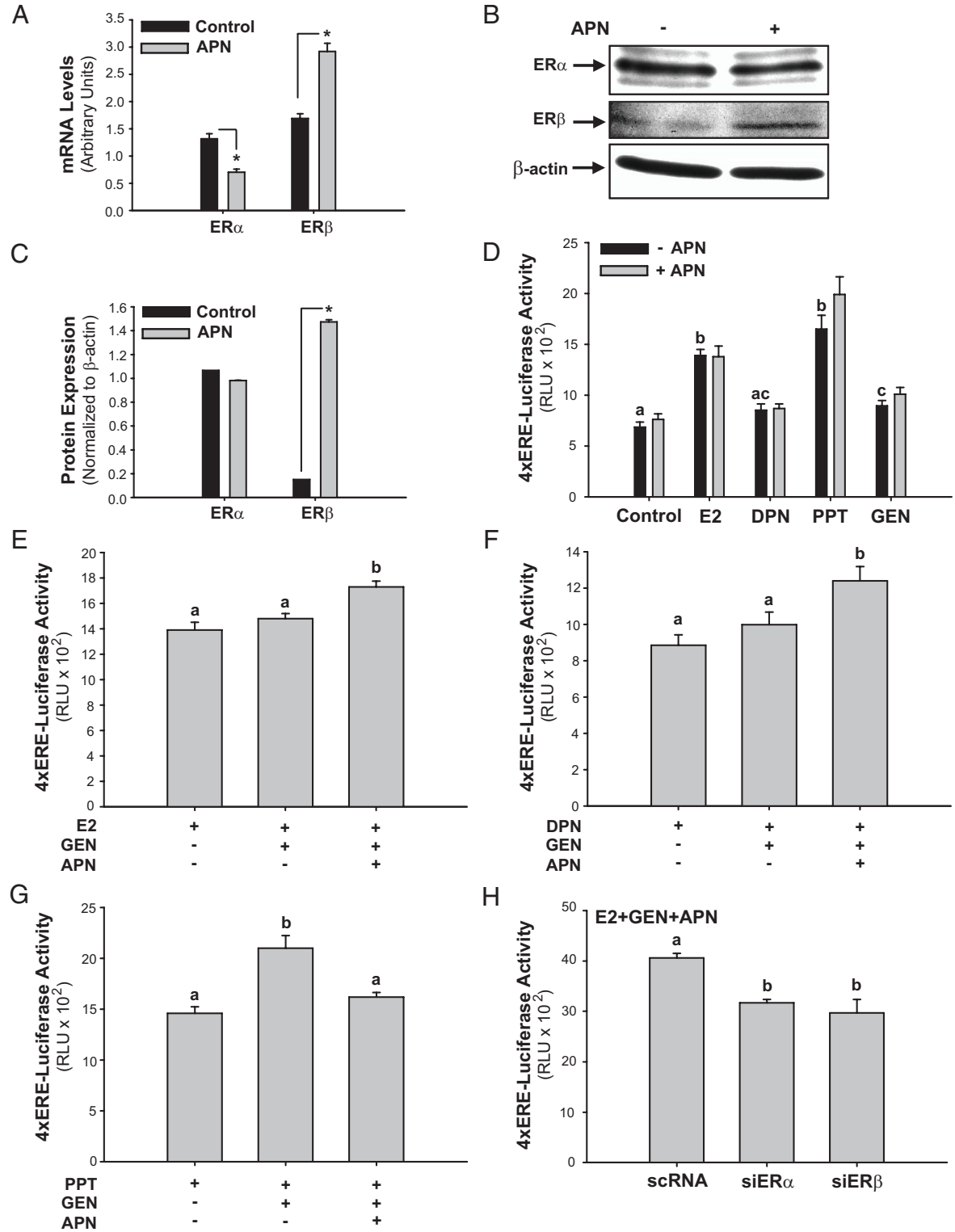

FIG. 4. APN induces ERβ expression and synergizes with GEN to promote ERβ transcriptional activity. A, Transcript levels of ERα and ERβ were quantified by QPCR in HC11 cells treated with APN (8 μg/ml) or vehicle (control) for 6 h. B, Western blot analysis of ERα and ERβ in lysates from cells treated as in A. *Each lane* contains 20 μg of total protein. C, Protein levels in B were quantified by densitometry and normalized to β-actin; *, $P < 0.05$ relative to control. D–H, HC11 cells were transfected with 0.5 μg of 4xERE-TK-Luc promoter/reporter construct in the presence or absence of control siRNA (scRNA) or siRNA targeting ERα or ERβ and treated with APN and specific ER ligands, alone and in combination. D, Cells were treated with vehicle (control), E_2, DPN, PPT, or GEN in the presence or absence of APN (8 μg/ml) for 24 h, and lysates were subsequently analyzed for luciferase activity. Effects of GEN and APN on the transcriptional response of E_2 (E), DPN (F), and PPT (G) were analyzed. H, Effects of knockdown of ERα and ERβ by respective siRNA on Luciferase reporter activity in cells treated with E_2 + GEN + APN were evaluated, relative to nontargeting (scRNA) siRNA. Values are means ± SEM from at least three independent experiments performed in triplicate. Means with different letters differed from control (D–G) or nontargeting scRNA (H) at $P < 0.05$. RLU, Relative luminescence unit.

requiring the availability of ER-specific ligands (*e.g.* GEN for ERβ) that can recruit specific coactivators and/or repressors (46).

GEN synergizes with APN to influence ligand-activated ERβ transcriptional activity

We have previously shown that GEN at physiologically relevant doses (40 nM, 2 μM) elicited increased differentiation, decreased proliferation, and promoted apoptosis of MEC *in vitro*, consistent with it being a major bioactive component of soy foods with breast cancer protective effects (50–52, 64). Our findings, thus far, suggest that *in vivo*, mammary stromal adipocytes and neighboring epithelial cells constitute coordinate targets of GEN action. To examine whether stromal adipocyte-derived APN promotes GEN action in MEC by enhancing GEN activation of ERβ signaling, we evaluated whether APN influences the transcriptional activity of GEN in the presence of the physiologically relevant ligand E_2 and compared these effects with those elicited with specific ERβ (DPN) and ERα (PPT) agonists. HC11 cells were treated with E_2 (10 nM), DPN (40 nM), or PPT (40 nM) in the presence or absence of APN (8 μg/ml) and/or GEN (40 nM), and 24 h later, 4xERE-Luc activity was analyzed. GEN in the absence of APN had no effect on basal ERE-TK-Luc activity of E_2 (Fig. 4E). Similar for E_2, GEN had no significant effect on DPN-activated promoter activity in the absence of APN (Fig. 4F). By contrast, GEN increased PPT-activated transcriptional responses in non-APN-treated cells (Fig. 4G). Interestingly, APN cotreatment with GEN significantly influenced these cells' transcriptional responses to all ligands (Fig. 4, E–G). In particular, APN enhanced Luc promoter activity in GEN + E_2-treated (Fig. 4E) and GEN + DPN-treated (Fig. 4F) cells while decreasing this activity in PPT + GEN-treated cells to PPT-alone levels (Fig. 4G).

Given that GEN preferentially binds ERβ, the above findings are consistent with GEN + APN promoting ERβ signaling by increasing ERβ homodimer (with DPN) or ERα/ERβ heterodimer (with E_2) transcriptional activities at the expense of ERα homodimer-mediated (with PPT) transactivity. To address this, ERα or ERβ expression was knocked down in APN + GEN + E_2-treated cells transfected with 4xERE-TK-Luc plasmid, using a pool of siRNA targeting each ER isoform. A decrease by approximately 25% in 4xERE-TK-Luc promoter activity was observed with ERα siRNA or ERβ siRNA added at equivalent concentrations (50 nM), relative to cells treated with nontargeting siRNA [scrambled RNA (scRNA), 50 nM] (Fig. 4H). We determined 50 nM as an optimal dose with effective knockdown (~70%) at the mRNA level (data not shown). Interestingly, the magnitude of the decrease in promoter activity achieved by targeting either ERα or ERβ with respective siRNA was comparable with the extent of promoter activity induction in APN + GEN + E_2-treated cells, when compared with those treated with E_2 + GEN in the absence of APN (Fig. 4E).

APN/GEN promotion of ERβ signaling is associated with up-regulation of proapoptotic and prodifferentiation gene expression

To determine whether APN promotion of GEN-mediated ERβ signaling may underlie the observed *in vivo* enhancement of MEC differentiation associated with exposure to SPI or GEN-supplemented diets, leading to mammary tumor protection (50–52, 55, 56, 64), we determined the expression of select proapoptotic (*Bad*, *p53*), antiapoptotic (*Bcl2*, *survivin*), and prodifferentiation/proapoptotic (*Pten*) genes in APN + GEN-treated cells cotreated with either E_2, DPN, or PPT. Because APN induced ERβ signaling in the absence (Fig. 4, A–C) or presence of E_2 (Fig. 4E), and in view of previous findings showing enhancement of cell proliferation by E_2 upon blockage of ERβ in HC11 cells (65), the activity of proapoptotic proteins caspase-3 and caspase-7 was initially evaluated in HC11 cells treated with E_2 alone, E_2 + GEN, and E_2 + GEN in the presence of APN, for 72 h. APN + E_2 + GEN cotreatments enhanced caspase-3/7 activity as measured by luminescence, compared with treatment with E_2 or E_2 + GEN (Fig. 5A). Further, cells cotreated with APN + GEN + E_2 showed increased *Pten* and decreased *survivin* expression, with no comparable changes in *Bad*, *Bcl2*, and *p53* transcript levels, when compared with non-APN-treated controls (Fig. 5B). Cells cotreated with DPN in the presence of GEN + APN elicited changes in gene expression levels consistent with APN + GEN inhibition of ERα signaling and promotion of ERβ-mediated transcriptional responses (Fig. 5C). In particular, transcript levels for *Bad*, *p53*, and *Pten* were increased, whereas those for *Bcl2* and *survivin* were attenuated with DPN + GEN + APN cotreatments. The transcript levels of *Bad* and *p53* were increased, and those for *Bcl2* were decreased, with no changes in *Pten*, or *survivin* expression levels, with PPT + GEN in the presence of APN, relative to without APN (Fig. 5D). Taken together, our results show that the concerted actions of APN and GEN in the promotion of ERβ signaling under physiological E_2 levels may underlie the contribution of adiposity/diet interactions to influence mammary epithelial differentiation and apoptosis for breast cancer prevention.

Discussion

In this study, we present a novel mechanism for dietary regulation of ERβ signaling in MEC involving the adipo-

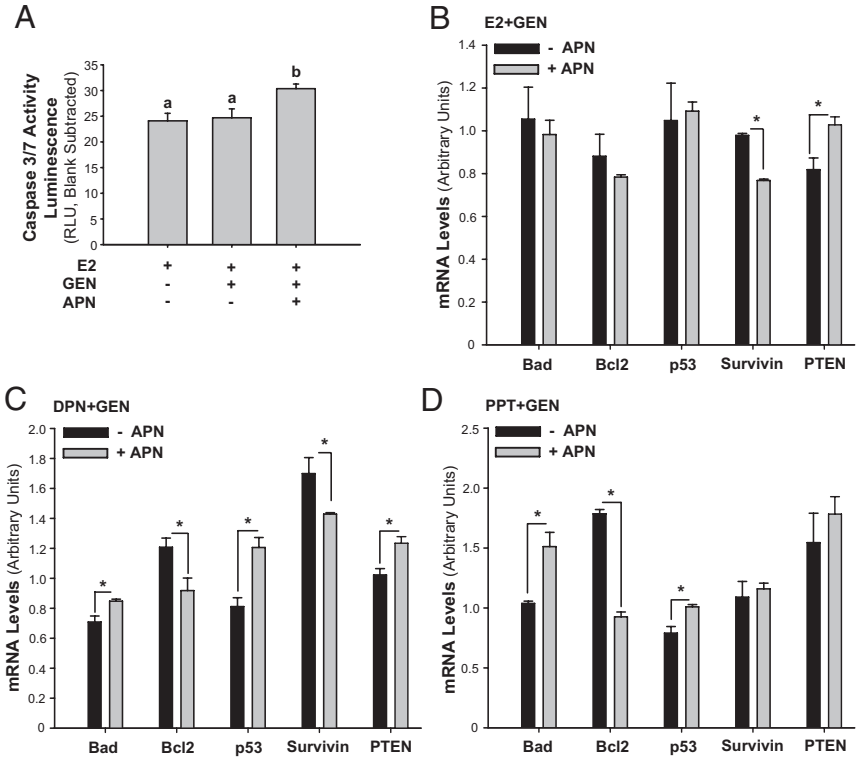

FIG. 5. Synergistic actions of APN and GEN enhance apoptosis in MEC. A, HC11 cells were treated with E_2 (10 nM) in the presence or absence of GEN (40 nM) and/or APN (8 μg/ml) for 72 h and subsequently assessed for caspase-3/7 activity, as described under *Materials and Methods*. Means with different letters differed from control at $P < 0.05$. Representative graph from two independent experiments, each performed in quadruplicate is shown. B–D, Gene expression of proapoptotic, differentiation-related (Bad, p53, and PTEN), and antiapoptotic (Bcl2, survivin) proteins was quantified by QPCR in cells treated with E_2 + GEN (B), DPN + GEN (C), and PPT + GEN (D) in the presence (+APN) or absence (−APN) of APN for 24 h. *Tbp* was used as a normalizing control; *, $P < 0.05$ relative to (−) APN group. Bad, Bcl2-Associated agonist of cell death; p53, tumor suppressor p53; Bcl2, B-cell leukemia/lymphoma 2; RLU, relative luminescence unit.

kine APN, whose attenuated expression with higher adiposity is associated with increased breast cancer risk. We provide evidence to support a model (Fig. 6) in which dietary induction of APN protein levels in the mammary stromal adipocytes induces ERβ expression in MEC to facilitate synergistic activation of ERβ signaling by the ERβ-selective ligand GEN in concert with the physiologically relevant ligand E_2, to inhibit proliferation, enhance differentiation, and promote apoptosis of MEC for breast cancer prevention. We further show that in MEC lacking functional ER, simulating those in prepuberty or postmenopausal conditions, APN functions in an ER-independent manner by inhibiting STAT3 activation, an event associated with increased epithelial differentiation as measured by mammary acini formation. Together, our findings provide a linear pathway by which diet-regulated mammary adipocyte APN and, hence, mammary adiposity status may modify the prepubertal/peripubertal and perimenopausal/postmenopausal mammary epithelium to achieve enhanced differentiation and integrate the local stromal adipocyte environment in orchestrating ER-dependent and ER-independent mechanisms to promote neighboring epithelial resistance to tumorigenic agents (66).

A major novel finding of the present study is the robust local induction of APN protein in mammary tissue by dietary intake of soy protein at peripuberty, a critical stage of mammary gland development. Although decreased serum APN levels are associated with increased risk and aggressiveness of breast cancer (12–14), a detailed role for local mammary tissue-derived APN in the promotion of breast cancer has not been fully established. Our current and previous (54) analyses of mammary glands of peripubertal rats from dams consuming SPI diets at amounts modeling the regular intake of soy-rich foods by the Asian population demonstrate the impact of early maternal diet on the adiposity of the developing mammary gland. Further, our identification of APN as a diet-regulated mammary adipocyte-secreted protein that mediates differentiation events in normal (nontumorigenic) ER-positive (HC11) and ER-negative (MCF-10A) MEC has important implications for the suggested intrauterine origins of breast cancer risk. In support of the latter, recent findings implicate a role for maternal obesity in the programming of APN signaling in offspring (67) and for systemic APN in children as a presumptive determinant of obesity-related diseases in later adult life (68–70).

Another novel finding from this study is the demonstration of APN/ER cross talk, leading to the promotion of ERβ transcriptional activation in normal MEC. Although a connection between APN and ER signaling in breast cancer cells has been previously raised (63), the current study takes a further step by addressing the influence of the dietary bioactive factor GEN in APN/ER cross talk in normal (nonbreast cancer) epithelial cells. We showed that APN in the presence of GEN modified the direction of genomic ER signaling by two mechanisms. First, APN preferentially increased ERβ isoform expression in HC11 cells, resulting in a higher ratio of ERβ to ERα at the levels of transcript and protein. Second, APN in concert with GEN altered the transcriptional responses of HC11 cells

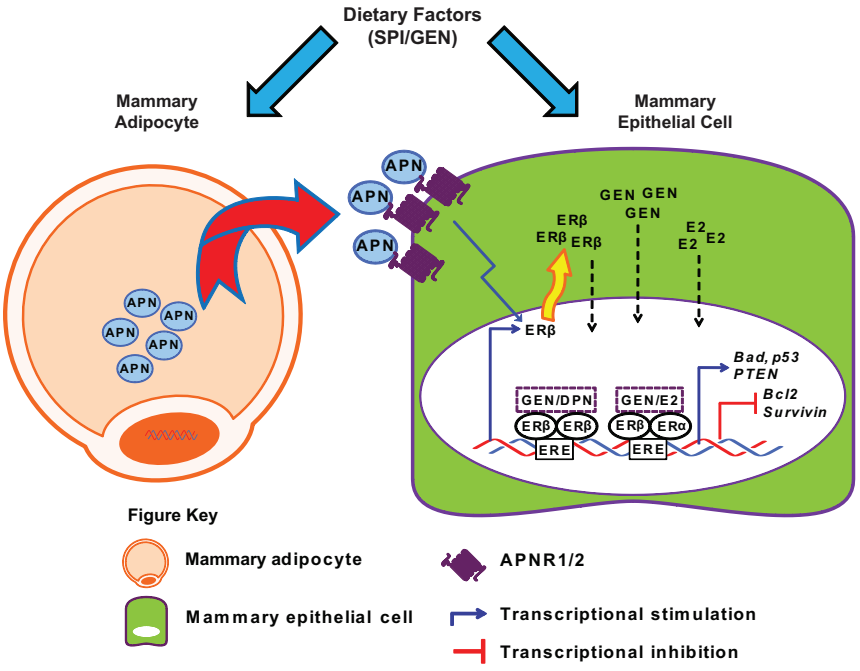

FIG. 6. Proposed model for synergistic actions of mammary adipocyte-derived APN and GEN on ERα/β cross talk to influence MEC proliferation, apoptosis, and differentiation. Dual targeting of mammary stromal adipocytes and MEC by dietary factors (*e.g.* soy isoflavone GEN) results in paracrine signaling between these two compartments. Dietary factor (SPI and/or GEN) induction of APN production by the mammary adipocyte initiates APN paracrine action on neighboring MEC expressing APNR, to induce ERβ expression and ERβ transcriptional activity in synergy with the ERβ-selective ligand GEN. Biological outcomes of this signaling include inhibition of ERα-mediated cell proliferation and enhancement of differentiation and apoptosis (up-regulation of *Bad*, *p53*, *PTEN*; down-regulation of *Bcl2*, *survivin*), all of which are hallmarks of decreased breast cancer risk.

to E_2 and pure ERα and ERβ agonists. With E_2, which normally induces formation of ERα homodimers and ERα/β heterodimers, and with PPT, which induces solely ERα homodimers, the presence of APN + GEN promoted the formation of ERα/β heterodimers and/or ERβ homodimers at the expense of ERα homodimers, thus enhancing GEN-mediated ERβ activation. Although we did not quantify the magnitude of the shift from ERα to ERβ signaling with APN + GEN cotreatments, the functional outcome of increased ERβ signaling was manifested as higher expression of proapoptotic and prodifferentiation genes, both of which are hallmarks of decreased breast cancer risk. The demonstration that *Pten* expression was similarly induced by APN + GEN in concert with E_2 or DPN but not PPT suggests *Pten* as a gene target of ERβ transactivation in MEC. Further, the induction of *Bad*, *Bcl2*, and *p53* expression by APN + GEN + DPN and the inhibition of their respective expression by APN + GEN + PPT reflect the opposing effects of ERβ on ERα transcriptional activity. These findings are consistent with ERβ attenuation of ERα-mediated transcriptional activation (34–42, 43) and the negative consequence of ERβ signaling on ERα-enhanced epithelial proliferation (65). Importantly, these results support the notion that mammary adiposity (using APN as a measure) regulates the balance of ERα *vs.* ERβ signaling in the presence of selective estrogen modulators, to alter MEC phenotype. Although it is also possible that APN action may occur via nonclassical (ligand independent) mechanisms involving MAPK-induced ER phosphorylation (71), we believe that this is unlikely, because APN effects on induction of ERβ transcriptional activity occurred only in synergy with the selective ERβ ligand GEN. Our findings that APN elicited a decrease in ERα transcript but not protein levels raise the possibility of distinct transcriptional and posttranslational regulation by APN of ER proteins; however, to our knowledge, this has not been previously reported.

An important feature of APN that was additionally demonstrated in the present study is the ability of this adipokine to induce mammary epithelial differentiation by a mechanism independent of ER signaling. Specifically, in ER-negative MCF-10A cells, APN promotion of epithelial differentiation was associated with its inhibition of activated STAT3 signaling as measured by decreased levels of phosphorylated STAT3. These results are consistent with previous studies documenting multiple proproliferation signaling cascades blocked by APN (58) and underscore the broad implications of dysfunctional adipose tissue on numerous metabolic, inflammatory, and chronic diseases. Further, given that the lack of ER signaling simulates that of prepuberty and postmenopausal status in women, these findings provide a mechanism by which adiposity can influence breast cancer risk.

In summary, we identified dietary regulation of mammary-specific APN production to occur at an early developmental window, and which may guide the direction of ligand-activated ER signaling in neighboring epithelial cells (Fig. 6). This model predicts that maintenance of dysfunctional adipose tissue elicited by an obesogenic state will have significant deleterious consequences to mammary breast health beginning at puberty. Our results also raise the intriguing (although yet untested) possibility that increased exposure to environmental agents with distinct selective ER modulator activities in the face of the obesity pandemic among children and young adults (72) may underlie, in part, the rising incidence of breast cancer worldwide.

Acknowledgments

We thank Dr. Frank A. Simmen and Dr. Stewart MacLeod (University of Arkansas for Medical Sciences) for insightful discussions during the course of this study.

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This work was supported by grants from the United States Department of Agriculture (CRIS 6251-5100002-06S, Arkansas Children's Nutrition Center), the University of Arkansas Children's University Medical Group, and the Fashion Footwear of New York (funded through University of Arkansas for Medical Sciences) (R.C.M.S.). O.M.R. is supported by the Department of Defense Breast Cancer Research Program Predoctoral Fellowship W81XWH-10-1-0047.

Disclosure Summary: The authors have nothing to disclose.

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Carcinogenesis vol.33 no.3 pp.652–660, 2012
doi:10.1093/carcin/bgr317
Advance Access publication January 4, 2012

Repression of mammosphere formation of human breast cancer cells by soy isoflavone genistein and blueberry polyphenolic acids suggests diet-mediated targeting of cancer stem-like/progenitor cells

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Mammary stem cells are undifferentiated epithelial cells, which initiate mammary tumors and render them resistant to anticancer therapies, when deregulated. Diets rich in fruits and vegetables are implicated in breast cancer risk reduction, yet underlying mechanisms are poorly understood. Here, we addressed whether dietary factors selectively target mammary epithelial cells that display stem-like/progenitor subpopulations with previously recognized tumor-initiating potential. Using estrogen receptor-positive MCF-7 and estrogen receptor-negative MDA-MB-231 human breast cancer cell lines and freshly isolated epithelial cells from MMTV-Wnt-1 transgenic mouse mammary tumors, we demonstrate that sera of adult mice consuming soy isoflavone genistein (GEN) or blueberry (BB) polyphenol-containing diets alter the population of stem-like/progenitor cells, as measured by their functional ability to self-renew and form anchorage-independent spheroid cultures *in vitro* at low frequency (1–2%). Serum effects on mammosphere formation were dose-dependently replicated by GEN (40 nM >2 μM) and targeted the basal stem-like CD44+/CD24−/ESA+ and the luminal progenitor CD24+ subpopulations in MDA-MB-231 and MCF-7 cells. GEN inhibition of mammosphere formation was mimicked by the Akt inhibitor perifosine and was associated with enhanced tumor suppressor phosphatase and tensin homologue deleted on chromosome ten (PTEN) expression. In contrast, a selected mixture of BB phenolic acids was only active in MDA-MD-231 cells and its CD44+/CD24−/ESA+ subpopulation, and this activity was independent of induction of PTEN expression. These findings delineate a novel and selective function of distinct dietary factors in targeting stem/progenitor cell populations in estrogen receptor-dependent and -independent breast cancers.

Introduction

Breast cancer, like many other human cancers, is considered to be derived from and maintained by a small population of self-renewing tumor-initiating cells, designated as cancer stem cells (CSCs) (1–5). Nonetheless, the origin of CSC remains to be fully elucidated. The long-held notion is that of a unidirectional hierarchical model wherein a pool of stem cells (SC) residing at the top of the epithelial hierarchy undergoes oncogenic transformations, selectively endowing these cells with tumor-initiating properties while maintaining their ability to generate more differentiated progeny lacking tumorigenic potential (1,6). In a recent study, Weinberg *et al.* presented strong evidence for the bidirectional interconversion of CSC and non-CSC in human mammary epithelial cell populations (7), thus expanding if not replacing the latter model. Although it is thought that CSC and normal SC share developmental programs that regulate critical SC fate and maintenance (8), the control of growth and differentiation-associated pathways in cells-of-origin of CSC likely diverged or is subverted from those of normal counterparts (9). In this regard, deregulation of Wnt, Notch, Hedgehog and phosphatase and tensin homologue deleted on chromosome ten (PTEN)/phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways, all of which constitute growth-control pathways for normal SC have been shown to underlie aberrant CSC self-renewal leading to breast cancer (10–13).

The incidence of breast cancer varies worldwide, a consequence in part of environmental rather than genetic differences and implicating dietary and lifestyle disparities among the general population (14). Prevailing evidence from epidemiological and experimental data suggest that breast cancer development can be influenced by diet/nutrition (15–18). Previous studies by our group (19,20) and others (21) have shown that dietary intake of soy-rich foods containing genistein (GEN) and GEN-supplemented diets inhibit chemically-induced mammary tumor formation in rodent models, in part through inhibition of Wnt signaling and upregulation of PTEN expression (22–25). Likewise, we have shown that in rodent models, early exposure to blueberry (BB) solely through maternal diet, enhanced mammary epithelial differentiation in prepubertal progeny, a process mediated by upregulation of PTEN expression and its nuclear localization (26). Given that PTEN and Wnt signaling pathways are functionally linked (27) and that these same pathways constitute key regulators of mammary epithelial SC biology (1,8,11,12), our findings raise the interesting possibility that SC, specifically CSC comprise viable targets of dietary factors.

Breast cancer cell lines, MCF-7 and MDA-MB-231, have been shown to display a subpopulation of cells with SC-like properties, defined experimentally by their ability to grow as spheroids in the absence of attachment and to self-renew in secondary or additional passages *in vitro* (3,5,28). These cell lines contain an ALDEFLUOR-positive population expressing the SC marker aldehyde dehydrogenase (29), which was previously used to isolate epithelial subpopulations displaying CSC properties from human breast tissues and breast carcinomas (30). Furthermore, flow cytometric analyses of MDA-MB-231 cells characterized a subpopulation displaying the $CD44^+/CD24^-/ESA^+$ phenotype, which when grafted to non-obese diabetic/severe combined immunodeficient mice at limiting dilution, rapidly formed tumors (28). The availability of these *in vitro* models in which CSC reside in a niche with more differentiated progeny approximating that *in vivo*, provide opportunities for addressing proof-of-concept questions of potential clinical significance for mitigating breast cancer relapse and drug resistance (1–4,31).

In the present study, we utilized MCF-7 and MDA-MB-231 cells to address the postulate that mammary stem/progenitor cells with tumor-initiating potential are direct targets of dietary components with known anti-breast cancer effects. MCF-7 is a well-differentiated estrogen receptor (ER)-positive breast cancer cell line, whereas MDA-MD-231 is a highly metastatic ER-negative cell line (32). To provide evidence for a role for dietary factors in limiting SC/progenitor cell-enriched populations, we evaluated sera from adult female mice consuming isocaloric and isonitrogenous diets containing GEN and BB, pure GEN as well as a select mixture of BB polyphenolic acids, for their ability to inhibit mammosphere formation in both cell lines. We show here that the inhibitory activity of sera from GEN-exposed animals was recapitulated by exogenous GEN in both cell lines and in freshly isolated epithelial cells from Wnt-1 transgenic (Wnt-Tg)

Abbreviations: BB, blueberry; CAS, casein; CSC, cancer stem cell; ER, estrogen receptor; GEN, genistein; PI3K, phosphatidylinositol 3-kinase; PR, progesterone receptor; PTEN, phosphatase and tensin homologue deleted on chromosome ten; SC, stem cell; Wnt-Tg, Wnt-1 transgenic.

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mouse mammary tumors (33). Furthermore, we show that GEN inhibition of mammosphere formation was associated with attenuated PI3K/Akt signaling and upregulated PTEN expression and targeted the $CD44^+/CD24^-/ESA^+$ subpopulation, previously shown to generate tumors in immunocompetent mice (28) as well as the luminal progenitor-enriched $CD24^+$ cells. In contrast, a select mixture of phenolic acids found in sera of BB-exposed animals inhibited mammosphere formation only in the more aggressive MDA-MB-231 cells and its $CD44^+/CD24^-/ESA^+$ subpopulation. Our findings indicate SC/progenitor cells as functional targets of dietary factors with breast cancer risk reduction activities and expand the repertoire of diet-mediated PTEN-associated pathways that may be exploited to avert the occurrence and relapse of ER-positive and ER-negative breast cancers.

Materials and methods

Animal studies

Animal studies were carried out under protocols approved by the Institutional Animal Care and Use Committee, University of Arkansas for Medical Sciences. Mice were housed in polycarbonate cages under conditions of 24°C, 40% humidity and a 12 h light–dark cycle. MMTV-Wnt-Tg mice (33) were obtained from Jackson Laboratory (Bar Harbor, ME). Wild-type female mice of the same strain and Wnt-Tg males were mated to generate wild-type and Wnt-Tg female offspring. Genotyping protocols were described previously (27). Adult wild-type females (3–6 months) were lifetime exposed (beginning at gestation day 4) to one of three semipurified isocaloric diets made according to the American Institute of Nutrition-93G formulation (19) and served as source of sera for *in vitro* treatments (described below). These diets are: (i) Casein (CAS), containing casein (New Zealand Milk Products, Santa Rosa, CA) as sole protein source; (ii) GEN, CAS supplemented with GEN aglycone at concentrations present in soy protein isolate (250 mg/kg food); and (iii) BB, CAS supplemented with 3% whole BB powder (FutureCeuticals, Momence, IL). Wnt-Tg females fed on CAS develop tumors within ages 6–8 months (27,33,34) and were used as source of mammary tumors for epithelial cell isolation (below). Tumors detected by palpation, were removed 2 weeks after initial appearance.

Cell lines and reagents

The human breast cancer cell line MCF-7 was purchased from American Type Culture Collection (Manassas, VA). The human MDA-MB-231 cell line was provided by Dr Thomas Kelly (University of Arkansas for Medical Sciences). Both lines were propagated in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (GIBCO, Carlsbad, CA) and 1% antibiotic–antimycotic solution (GIBCO). The MDA-MB-231 culture media additionally contained 10 μg/ml insulin (Sigma–Aldrich, St Louis, MO) and 2 mM glutamine (GIBCO). Cells were incubated in 5% CO_2:95% air at 37°C. GEN, dimethyl sulfoxide, Akt inhibitor perifosine, hippuric acid, 3-hydroxyphenylacetic acid, 3-hydroxybenzoic acid, ferulic acid, 3-(4-hydroxyphenyl) propionic acid and 3-hydroxycinnamic acid were obtained from Sigma–Aldrich. 3-(3-Hydroxyphenyl) propionic acid was purchased from Alfa Aesar (Ward Hill, MA).

Mammosphere formation assay

MCF-7 and MDA-MB-231 cells, when seeded in ultra-low attachment plates (Corning, Corning, NY) in serum-free media form non-adherent spheroids termed mammospheres, with the ability to self-renew (28). Plating medium for mammosphere formation consisted of phenol red-free serum-free Minimal essential media, supplemented with B27 (1; Invitrogen), 20 ng/ml human basic fibroblast growth factor (Invitrogen), 20 ng/ml human epidermal growth factor (Invitrogen), 10 μg/ml heparin (Sigma–Aldrich), 1% antibiotic–antimycotic solution (Invitrogen) and 100 μg/ml gentamicin (Sigma–Aldrich). To examine the effects of various treatments on mammosphere formation, MCF-7 and MDA-MB-231 cells were seeded in 24-well ultra-low attachment plates in plating medium with and without added treatments at a density of 2500 cells per well. Plating medium was refreshed every 3 days in the absence of additional treatments and the appearance of primary spheres (P1) was evaluated after 5 days. Mammospheres with diameters of $\geq$100 μm (MCF-7) and $\geq$60 μm (MDA-MB-231) were manually counted using a Carl Zeiss Axiovision microscope (Carl Zeiss AG, Oberkochen, Germany). To assess the relative sphere numbers over second (P2) and third (P3) passages, mammospheres from the previous plating were collected at day 5 (P1) or 7 (P2), dissociated with 0.05% trypsin (Invitrogen) into single-cell suspensions, filtered using a 40 μM sieve and replated in ultra-low attachment plates, with no additional treatments. Treatment effects were determined from at least three independent experiments in quadruplicates.

Mammary tumor epithelial cells, treatments and mammosphere formation assay

The isolation of mouse mammary tumor epithelial cells followed previously described protocols (34,35). Briefly, mammary tumors removed from Wnt-Tg females were incubated in digestion medium [Dulbecco's modified Eagle medium/F12 containing 100 μg/ml gentamicin, 1% antibiotic–antimycotic and Collagenase Type III (225 units/ml/g tissue; Worthington, Lakewood, NJ)] at 37°C for 2.5 h in a rotary shaker at 125 r.p.m. Cells were filtered through 40 μm cell strainers, washed four times in washing buffer (Dulbecco's modified Eagle medium/F12, 5% fetal bovine serum and 50 μg/ml gentamicin) at 2000 r.p.m. for 2 s and then in phosphate-buffered saline. Subsequent evaluation of mammosphere numbers followed that described for the breast cancer cell lines (above), except that cells were plated in six-well low-attachment plates at a density of 10 000 (P1) and 5000 (P2) cells per well.

RNA extraction and quantitative real-time polymerase chain reaction

Total RNA was prepared from mammospheres collected at days 5 (P1) and 7 (P2) post-plating, using Trizol reagent (Invitrogen), subjected to RNase-free DNAse treatment and converted into complementary DNA by using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative real-time polymerase chain reaction analyses used SYBR Green and the ABI Prism 7000 Detection System (Applied Biosystems, Foster City, CA), and primer design was performed as described (25). The primers used for gene expression analyses are presented in Supplementary Table 1, available at *Carcinogenesis* Online. TATA-box binding protein messenger RNA was used as normalizing RNA and fold-change was calculated based on vehicle-treated normalized values for each transcript.

Fluorescence-activated cell sorting

The phenotypes of parental MCF-7 and MDA-MB-231 cells grown in plastic were determined by flow cytometry using a FACS Aria cell sorting flow cytometer (BD Biosciences) and human luminal (CD24-PE) and basal (CD44-APC) epithelial markers (BD Pharmingen, San Jose, CA). MDA-MB-231 cells were enriched for tumor-initiating cells by flow cytometry using CD44-APC, CD24-PE (both from BD Pharmingen) and ESA-fluorescein isothiocyanate (Stem Cell Technologies, Vancouver, Canada). Briefly, cells (1×10^7/ml) were resuspended in Hank's balanced salt solution containing 2% fetal bovine serum and 100 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid and stained with primary antibodies (1:100 dilution) or isotype controls for 15 min at room temperature. Cells with the $CD44^+/CD24^-/ESA^+$ phenotype were used for mammosphere formation at a seeding density of 10 000 (P1) and 5000 (P2) cells per well. P1 and P2 mammospheres were collected for gene expression analyses by quantitative real-time polymerase chain reaction.

Analyses of serum metabolites

Sera collected from adult female mice assigned to CAS- or BB-diets ($n = 6$ per dietary group) were analyzed for phenolic metabolites following previously published procedures (36).

Data analysis

The statistical significance of differences in numerical data was evaluated using SigmaStat version 3.5 for Windows. Data were analyzed using Student's *t*-test or one-way analysis of variance. A P value <0.05 was considered to be statistically significant.

Results

Mammosphere formation by MCF-7 and MDA-MB-231 cells

We initially characterized the size of the basal and luminal subpopulations within MCF-7 and MDA-MB-231 cell lines using specific antigens ($CD44^+$ for basal; $CD24^+$ for luminal). Consistent with a previous study (28), MCF-7 cells are highly enriched for the $CD24^+$ subpopulation, whereas MDA-MB-231 cells are predominantly $CD44^+$, with low to nil expression of CD24 (Figure 1a and b).

A small population of human breast cancer cells can survive and proliferate *in vitro* as floating spherical colonies under anchorage-independent conditions (5). These spheres, designated mammospheres given their mammary epithelial origin, exhibit SC-like/progenitor properties based on their ability to self-renew and initiate and/or sustain heterogeneous tumors. To confirm if the luminal epithelial-enriched, ER/progesterone receptor (PR)-positive MCF-7 and basal epithelial-enriched, ER/PR-negative MDA-MB-231 cells contain the SC/progenitor population as reported previously (28), cells were seeded in ultra-low attachment plates and spheres formed from the

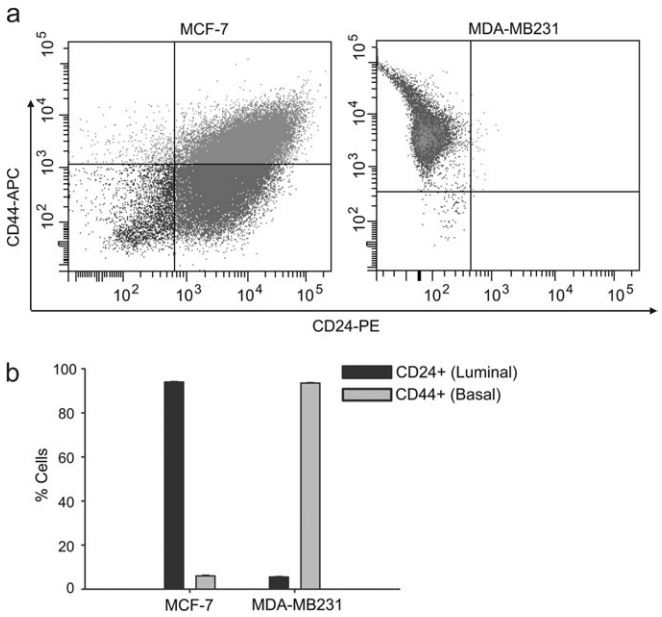

Fig. 1. Distinct epithelial phenotypes of human MCF-7 and MDA-MB-231 breast cancer cell lines. (**a**) Representative fluorescence-activated cell sorting analysis of MCF-7 and MDA-MB-231 cells using specific surface antigens for basal (CD44-APC) and luminal (CD24-PE) epithelial cells. In all experiments, cells were gated with isotype controls, following previously published protocols (28). (**b**) Summary of the percentage of cells positive for CD24 and CD44 for each cell line from $n = 3$ independent experiments.

original plating (P1) were collected and examined for subsequent formation of mammospheres after replating (P2 and P3). Both cell lines showed a small subset (1–2%) of the epithelial population capable of forming mammospheres upon initial plating (P1; Figure 2a and b). Mammospheres formed from MCF-7 cells tended to be bigger (≥100 μm on average) than those of MDA-MB-231 cells (∼60 μm on average). The percent of mammospheres formed was significantly increased from P1 to P2 for each cell line, suggesting enrichment of a subpopulation with self-renewal capacity. However at P3, mammosphere formation was either maintained at the P2 level (MCF-7) or reduced to that of P1 (MDA-MB-231); this is consistent with decreased self-renewal capacity of luminal progenitor cells with serial passage (37). Thus, we considered P1 and P2 mammospheres of both cell lines to contain a mixture of basal stem and luminal progenitor cells; further analyses were conducted with these cell passages.

Mammosphere formation by MCF-7 and MDA-MB-231 cells is inhibited by sera from mice consuming dietary GEN and BB

Diet and dietary factors modify breast cancer risk as shown in animal models and epidemiological studies (16–21). To investigate whether dietary factors can potentially target breast cancer cells with stem/progenitor properties *in vivo*, we conducted *ex vivo* studies wherein sera pools from adult female mice ($n = 6$ per group) fed CAS, GEN and BB were added at 1 and 5% (vol/vol) final concentrations (in mammosphere plating medium) to MCF-7 and MDA-MB-231 cells upon seeding in ultra-low attachment plates. Mammosphere formation at P1 was quantified 5 days later. Although MDA-MB-231 cells had a 10-fold lower efficiency than MCF-7 cells to generate mammospheres in suspension culture, sphere formation in both cell lines was significantly decreased by GEN-sera and BB-sera relative to CAS-sera at the

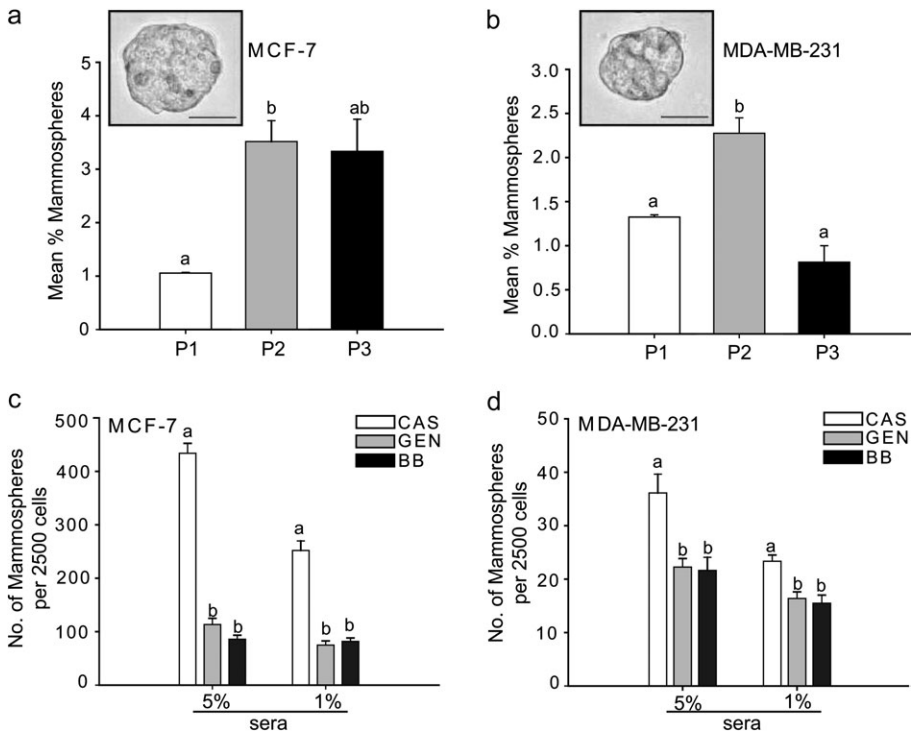

Fig. 2. Human breast cancer lines form mammospheres that are sensitive to factors found in sera of mice consuming various diets. (**a**) MCF-7 and (**b**) MDA-MB-231 cell lines were seeded to form primary mammospheres (P1) at a density of 2500 cells per well in 24-well low-attachment plates. After 5 days, P1 mammospheres were counted, collected and replated under the same conditions at a density of 1000 cells per well to form secondary mammospheres (P2). Data for P3 were obtained from P2. Mammospheres formed (% mean ± SEM) are expressed relative to number of cells plated for each passage and are from three independent experiments. Mean values with different letter subscripts differed at $P < 0.05$. Inset: Primary mammospheres formed from unsorted MCF-7 and MDA-MB-231 cells in suspension culture for 5 days. Magnification = ×100, scale bar = 50 μm. (**c** and **d**) Addition of sera from mice exposed to GEN- and BB-diets at 5 and 1% (v/v) final concentrations to plating medium, significantly reduced mammosphere formation of MCF-7 and MDA-MB-231 relative to control CAS diet. P1 spheres were counted at day 5 after plating. Data represent the number of mammospheres per 2500 plated cells (mean ± SEM) for each serum concentration. Values with different letters (a, b) differed at $P < 0.05$. $N = 3$ independent experiments, with each experiment carried out in quadruplicates.

concentrations tested (Figure 2c and d). The reduction in the numbers of mammosphere-initiating capacity elicited by GEN-sera and BB-sera relative to CAS-sera was more dramatic for MCF-7 (60–80%) than for MDA-MB-231 (40%) cells for the same serum concentration (Figure 2c and d).

Soy isoflavone GEN attenuates mammosphere formation by breast cancer cell lines and mammary tumor epithelial cells

GEN is bioavailable in humans and rodents consuming soy foods (17,18,38,39) and may underlie the decreased breast cancer risk attributed to dietary soy intake. To examine if GEN treatment limits mammosphere-forming ability, MCF-7 and MDA-MB-231 cells were treated at plating with GEN within the concentration range (40 nM and 2 μM) found in sera of regular soy food consumers (17,38,39) and evaluated for numbers of mammospheres formed at P1 and P2. GEN attenuated mammosphere formation in both cell lines at P1 and P2, with the lower dose (40 nM) eliciting consistently greater inhibitory effects than the higher (2 μM) dose (Figure 3a and b). Interestingly, although GEN at 2 μM dose was only effective in P2 mammospheres generated from MCF-7 cells, MDA-MB-231 cells were comparably insensitive to 2 μM GEN at P1 and P2.

Only a small subset of MCF-7 and MDA-MB-231 cells, specifically those characterized by the $CD44^+/CD24^-/ESA^+$ phenotype, exhibits bonafide CSC-like properties based on xenograft transplantation (28). To address whether GEN specifically target this subpopulation, MDA-MB-231 cells were isolated by fluorescence-activated cell sorting using fluorophore-labeled antibodies to CD44, CD24 and ESA; this isolation procedure resulted in a 2.5–5% yield of the $CD44^+/CD24^-/ESA^+$ subpopulation (data not shown). These cells were seeded at low density (P1, 2500 cells per well; P2,1000 cells per well) in ultra-low attachment plates and examined for GEN effects on mammosphere formation. GEN at 40 nM and 2 μM concentrations suppressed mammosphere formation in P1 and this effect was maintained in P2 (Figure 3c).

Transgenic expression of Wnt-1 in the mouse mammary gland (Wnt-Tg mouse) leads to lobuloalveolar hyperplasia and progression to carcinoma (33). Furthermore, enhanced Wnt-1 signaling promotes expansion of normal mammary SC and facilitates tumor formation by CSC (34). To demonstrate that Wnt-Tg mammary tumors contain an epithelial subpopulation with SC-like properties that may constitute targets of dietary factors *in vivo*, freshly isolated epithelial cells from mammary tumors of Wnt-Tg mice were examined for their ability to form mammospheres. Similar to the established cell lines, isolated cells formed mammospheres and were capable of self-renewal when evaluated at primary and secondary passages. GEN (40 nM) decreased the number of mammosphere-forming units in primary (data not shown) and in secondary (by 40–50% compared with control cells) passages (Figure 3d). The higher GEN dose (2 μM) showed no inhibitory effect on mammosphere formation when compared with control (vehicle only) treatment.

GEN inhibition of mammosphere formation involves PI3K/Akt signaling

Studies suggest that the PTEN/PI3K/Akt signaling pathway is involved in tissue-specific SC self-renewal (11). To evaluate whether GEN inhibits expansion of cells with self-renewal capabilities via this pathway, MCF-7 cells were treated with the Akt inhibitor perifosine (Akt-I, 0.5 μM) and GEN (40 nM), alone and together, at plating. Addition of Akt-I dramatically suppressed (by 70%) primary mammosphere formation in MCF-7 cells; this effect was effectively mimicked by 40 nM GEN (Figure 4a). Cells cotreated with 40 nM GEN + 0.5 μM Akt-I did not differ in mammosphere formation activity from those of cells treated with either GEN or Akt-I alone.

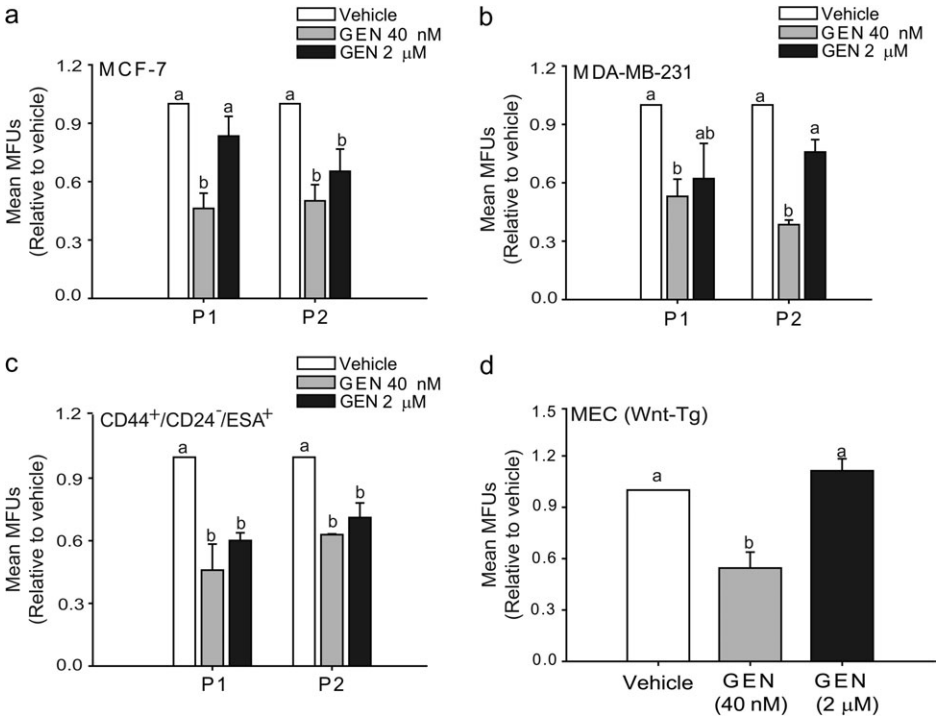

Fig. 3. GEN decreases the number of mammosphere-forming units (MFUs) in human breast cancer cell lines and in freshly isolated epithelial cells from mouse mammary tumors. (**a**) MCF-7 and (**b**) MDA-MB-231 cells were treated with GEN (40 nM and 2 μM) in 1% dimethyl sulfoxide or vehicle control (1% dimethyl sulfoxide) added to plating medium in low-attachment plates. Primary (P1) and secondary (P2) mammospheres were counted at 5 and 7 days, respectively after plating. Values are mean MFU ± SEM, normalized to those of control (vehicle only)-treated cells from three independent experiments. (**c**) GEN (40 nM and 2 μM) added to plating medium only at initial plating diminished the numbers of P1 and P2 mammospheres formed from $CD44^+/CD24^-/ESA^+$ subpopulation isolated from MDA-MB-231 cells, relative to control (vehicle only)-treated cells. (**d**) Freshly isolated epithelial cells from mammary tumors of Wnt-Tg mice were treated with GEN (40 nM and 2 μM) added to plating medium only at initial plating. P1 mammospheres were collected and passaged for P2 without additional GEN treatment. Data (mean MFU ± SEM) are from P2 mammospheres of two independent experiments. Each experiment utilized mammary tumors from different mice. Means with different letters (a, b) differed at $P < 0.05$.

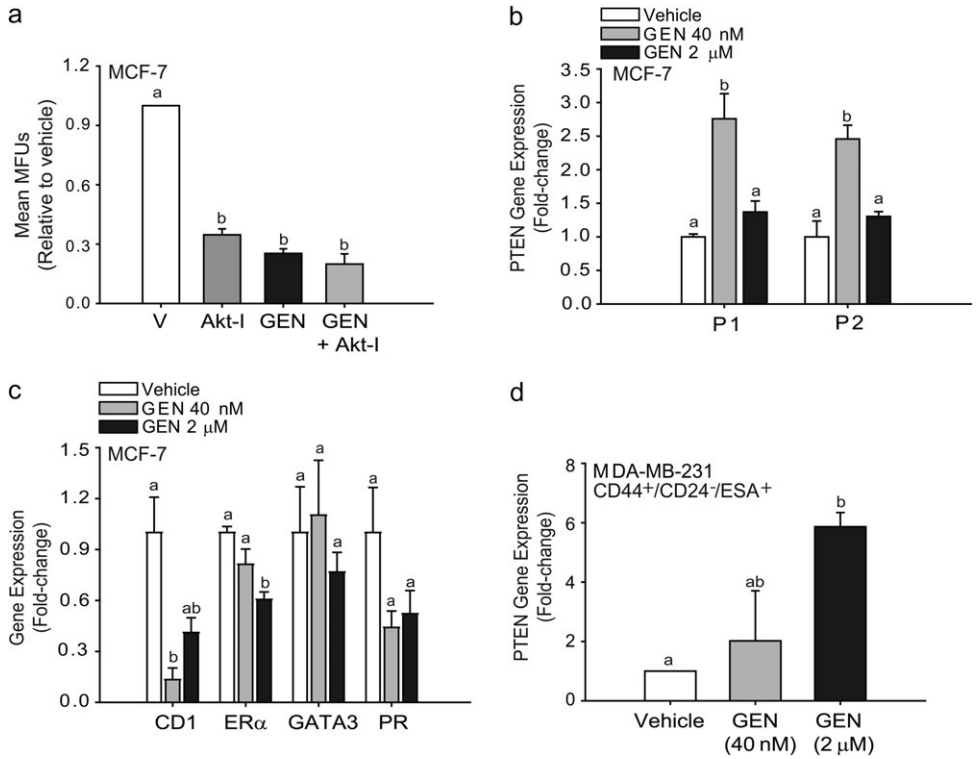

Fig. 4. GEN inhibition of mammosphere formation by mammary epithelial cells is associated with PTEN/PI3K/Akt regulatory pathway. (**a**) MCF-7 cells treated with Akt inhibitor perifosine (Akt-I) and GEN (40 nM), alone and together or vehicle alone, were evaluated for numbers of P1 mammospheres formed. Data are mean MFU ± SEM, relative to those of vehicle-only treated cells ($n = 3$ independent experiments). Means with different letters (a,b) differed at $P < 0.05$. (**b**) GEN induction of PTEN expression (quantified by quantitative real-time polymerase chain reaction) in P1 and P2 mammospheres is dose-dependent, with 40 nM showing a greater effect than 2 μM. Means with different letters (a, b) differed at $P < 0.05$ ($n = 3$ independent experiments), relative to vehicle-treated cells. (**c**) Transcript levels of cyclin D1 (CD1), ERα, GATA3 and PR in P1 mammospheres of MCF-7 cells treated with GEN (40 nM and 2 μM in 1% dimethyl sulfoxide) or vehicle alone (1% dimethyl sulfoxide). Transcript levels were quantified by quantitative real-time polymerase chain reaction and normalized to TATA-box binding protein and then renormalized to control (vehicle) values. Means with different letters (a, b) differed at $P < 0.05$. (**d**) Transcript levels of PTEN in P2 mammospheres formed from $CD44^+/CD24^-/ESA^+$ subpopulation of MDA-MB-231 cells were increased by GEN treatment. Means with different letters (a, b) differed at $P < 0.05$, relative to vehicle-treated cells ($n = 2$ independent samples).

PTEN is a tumor suppressor that antagonizes the PI3K/Akt pathway (40) and whose expression and activity are up-regulated by GEN in mammary epithelial cells (22,25). To investigate the potential participation of GEN-induced PTEN expression in the repression of mammosphere formation, *PTEN* expression levels were evaluated by quantitative real-time polymerase chain reaction in mammospheres collected from P1 and P2 after initial treatment of MCF-7 cells with 40 nM and 2 μM GEN. P1 mammospheres from cells treated with 40 nM but not with 2 μM GEN demonstrated significant upregulated *PTEN* expression, relative to vehicle-only treated cells (Figure 4b). This effect was maintained in P2 mammospheres. To confirm the specificity of the *PTEN* induction by the lower GEN dose, expression of other genes was quantified in P1 mammospheres. The transcriptional response of P1 mammospheres to the high and low doses of GEN was comparable for cyclin D1 (lower), GATA3 (no effect) and PR (numerically lower) (Figure 4c). In contrast, the inhibitory effect of GEN on ER-α transcript levels was specific to the higher dose (2 μM). *PTEN* expression was similarly analyzed in P2 mammospheres formed by the fluorescence-activated cell sorting-isolated $CD44^+/CD24^-/ESA^+$ subpopulation of MDA-MB-231 cells after a single treatment with GEN at initial plating. The higher GEN dose (2 μM) significantly increased *PTEN* expression in this subpopulation, whereas the lower dose was not as effective (Figure 4d).

BB phenolic acid mixture selectively inhibits mammosphere formation by MDA-MB-231 cells

Given our findings (Figure 2c and d) that sera from mice consuming dietary BB inhibited mammosphere formation in MCF-7 and MDA-MB-231 cell lines, we evaluated the effects of BB phenolic acids on mammosphere formation *in vitro.* We utilized an artificial mixture of the seven phenolic acids, namely hippuric acid, 3-hydroxyphenylacetic acid, 3-hydroxybenzoic acid, 3-(3-hydroxyphenyl)-propionic acid, ferrulic acid, 3-(4-hydroxyphenyl)-propionic acid and 3-hydroxycinnamic acid, based on a previous analyses of these compounds in sera of rats fed on 10% BB-diets (36). A range of concentrations (designated 5×, 10× and 20×) higher than those found in sera of rats fed on BB diet was used for the treatments to take into account the potential for under-estimation of the bioactive phenolic acid concentrations *in vivo* due to their ability to be metabolized via conjugation with glucoronides or sulfates and which may render them inactive (41). Addition of the phenolic acid mixture to MDA-MB-231 cells suppressed primary mammosphere formation (P1) in a dose-dependent manner, with the lower doses (5× and 10×) showing greater inhibitory effects than the highest dose (20×), which had no activity relative to vehicle control (Figure 5a). The inhibitory effects of the mixture at the two lowest concentrations (5 and 10×) were sustained in P2 mammospheres. Interestingly, the highest dose (20×) showed comparable inhibitory activity as the lower doses on mammosphere formation upon secondary passage.

Hippuric acid is the most abundant phenolic acid in sera of rats exposed to dietary BB (36). We measured its concentration in sera of mice exposed to BB-diets and found this to be 2.84 ± 0.33 μg/ml ($n = 3$ independent samples), which was ∼36-fold higher than in sera of mice fed on control CAS diet (0.078 ± 0.004 μg/ml). Analyses of BB-sera for the other six phenolic acids present in the artificial mixture showed levels that were considerably lower than those found for hippuric acid and did not significantly differ from those found in

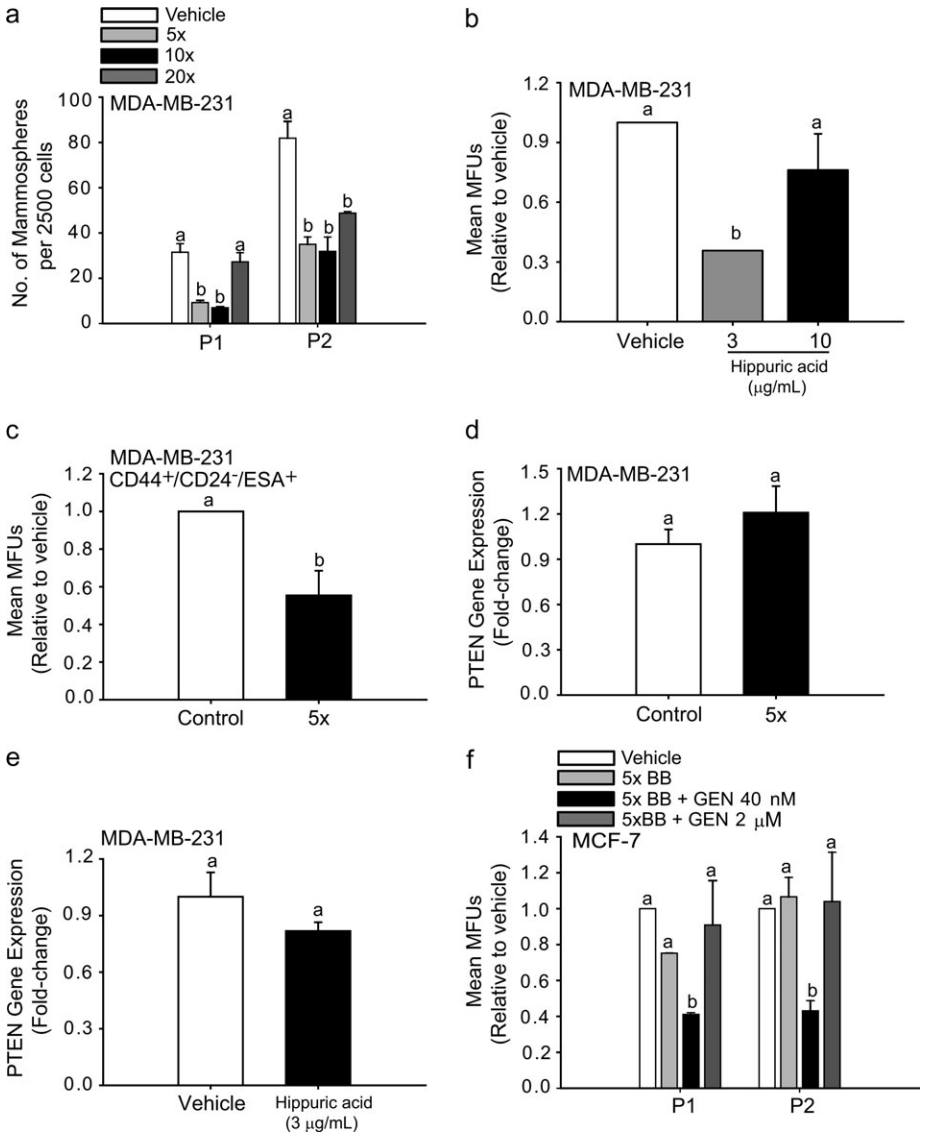

Fig. 5. BB polyphenolic acids influence mammosphere formation of mammary epithelial cells. (**a**) Different concentrations of an artificial mixture of seven polyphenolic acids present in sera of rats fed on 10% BB (described under Materials and methods) were added to MDA-MB-231 cells. The designations of 5×, 10× and 20× refer to the fold-concentrations of each metabolite found in sera of 10% BB-fed rats (36). Cells treated at initial plating were evaluated for primary (P1) and secondary (P2) mammospheres. Data are mean MFU ± SEM, relative to those of vehicle-only treated cells ($n = 3$ independent experiments). Means with different letters (a,b) differed at $P < 0.05$ for each passage. (**b**) The effect of hippuric acid on mammosphere formation of MDA-MB-231 cells was evaluated at two doses. Data presented as mean MFU ± SEM, relative to control cells, are from P1 of three independent experiments, with each experiment performed in quadruplicate. Means with different letters (a, b) differed at $P < 0.05$. (**c**) Addition of BB polyphenolic acid mixture (at 5× concentration) to $CD44^+/CD24^-/ESA^+$ subpopulation of MDA-MB-231 cells inhibited P1 mammosphere formation. Mean MFU ± SEM, relative to control cells, are from two independent experiments, with each experiment performed in quadruplicate. Means with different letters (a, b) differed at $P < 0.05$. (**d** and **e**) PTEN transcript levels were measured by quantitative real-time polymerase chain reaction in P1 mammospheres of MDA-MB-231 cells treated with vehicle and BB phenolic acid mixture (d) or vehicle and hippuric acid (e). Data are from two independent experiments, with each experiment carried out in triplicate. (**f**) Lack of combinatory effects of BB phenolic acid mixture and GEN on P1 and P2 sphere-forming capacity of MCF-7 cells. Cells were treated with 5× BB, alone or with GEN (40 nM and 2 μM) or with vehicle in three independent experiments, with each experiment carried out in triplicates. The numbers of mammospheres formed were expressed relative to control (vehicle-treated) cells. Mean MFU ± SEM) with different letters (a, b) differed at $P < 0.05$ for each passage.

CAS-sera (data not shown). We then evaluated the effect of hippuric acid on mammosphere formation at 3 μg/ml and 10 μg/ml, which represent physiological and supra-physiological doses, respectively. Although a 50% decrease in primary mammosphere formation was shown with the lower dose of hippuric acid, the higher dose was less effective and showed no significant inhibitory activity relative to control (Figure 5b).

Similar to that shown with GEN treatment (Figure 3c), mammosphere formation by $CD44^+/CD24^-/ESA^+$ subpopulation of MDA-MB-231 cells was diminished (by 50%) with the addition of the BB phenolic acid mixture (5× dose). However, mammosphere formation by MCF-7 cells was not affected by the mixture at all doses examined (data not shown), despite the demonstrated effects of BB-sera on these cells (Figure 2c). Furthermore, the mixture at 5× concentration had no effect on mammosphere formation by epithelial cells derived from mammary tumors of Wnt-Tg mice (data not shown). Unlike that of GEN, the inhibitory effects of BB phenolic mixture and hippuric acid on mammosphere formation by MDA-MB-231 cells were not accompanied by increased PTEN expression (Figure 5d and e). Co-addition of BB phenolic mixture (5×) and GEN demonstrated the mammosphere-inhibitory activity of 40 nM GEN and the lack of effect of 2 μM GEN, respectively with (Figure 5f) and without (Figure 2c) added 5× BB in MCF-7 cells. The same cotreatments in MDA-MB-231 cells resulted in additive effects of GEN (40 nM) and 5× BB in inhibiting

mammosphere formation (data not shown), confirming the selective activity of BB phenolic mixture on MDA-MB-231 cells.

Discussion

In this report, we provide evidence indicating that bioactive factors in foods with putative antimammary tumor activities target a subpopulation of mammary epithelial cells with mammosphere-forming capacity characteristic of SC/progenitor cells. Our studies utilized two human breast cancer cell lines (MCF-7 and MDA-MB-231) with distinct cancer subtypes (32), a subpopulation of MDA-MB-231 cells ($CD44^+/CD24^-/ESA^+$) with previously characterized tumor-initiating properties (28) and primary epithelial cells isolated from mammary tumors of a well-accepted mouse model of mammary tumorigenesis (MMTV-Wnt-1; Wnt-Tg) that recapitulates salient features of the human disease (33,34). We showed that GEN, a major soy isoflavone, and hippuric acid, a metabolite of BB polyphenols, can effectively attenuate mammosphere formation *in vitro* at physiologically relevant doses, reflecting their potential inhibitory effects on CSC self-renewal and expansion *in vivo*. We further showed that inhibition of PI3K/Akt signaling, associated with upregulation of PTEN expression constitutes a relevant pathway by which CSC/progenitor cell behavior may be controlled by particular dietary factors. Together, our results suggest a novel mechanism by which progression of breast cancer in women may be attenuated by consumption of 'healthy' diets. Given that cancer cells with SC-like properties underlie resistance of breast tumors to radiation and chemotherapeutic agents and hence increased tumor recurrence (2,4,5,31), our findings may have important implications for the design of dietary interventions for improving breast cancer outcomes.

The human breast cancer cell lines used in the present study (MCF-7 and MDA-MB-231) have been previously demonstrated to exhibit expression of the well-accepted SC marker aldehyde dehydrogenase expression, as assessed by the ALDEFLUOR assay (29, 30). Although we did not utilize this marker for isolating the epithelial subpopulation for mammosphere formation assays, the robust expression of $CD44^+/CD24^-$ antigens by the highly metastatic MDA-MB-231 and their corresponding lower expression in MCF-7 cells, as confirmed here, are consistent with the epithelial to mesenchymal transition traits associated with SC phenotype in ALDEFLUOR-positive cells (42). Furthermore, whereas freshly isolated mammary epithelial cells from Wnt-Tg tumors were not additionally fractionated for the $CD29^{hi}/CD24^{hi}$ subpopulation that was previously shown to display outgrowth potential using transplantation assay and hence SC properties (34), we found that these primary cells also exhibited reduced potential to self-renew in anchorage-independent conditions with low GEN dose (40 nM). This suggests the presence of an SC subpopulation in Wnt-Tg tumors that are responsive to GEN inhibitory activity. The collective findings establish MCF-7 and MDA-MB-231 cell lines as relevant *in vitro* models for large-scale screening of dietary and other factors for potential SC targeting activity to inform breast cancer therapy.

In previous studies (22,25), we showed that induction of PTEN expression and its increased nuclear localization are hallmarks of isoflavone GEN action in both non-tumorigenic mammary (e.g. MCF-10A) and breast cancer (MCF-7) cell lines. Increased PTEN expression in these cells resulted in decreased proliferative and increased apoptotic status, consistent with altered expression of genes involved in cell cycle arrest, survival and differentiation (22–25,43). The present studies suggest that GEN may act as a potent inhibitor of the expansion of cancer cells with SC/progenitor characteristics through its induction of PTEN expression in mammospheres. Although further studies will be required to mechanistically establish the functional association between GEN induction of PTEN expression and its ability to mimic the mammosphere-inhibitory activity of the Akt inhibitor perifosine [e.g. by PTEN small interfering RNA-mediated knockdown and measurement of activated (phosphorylated) AKT protein levels], these findings have important implications given that the PTEN/PI3K/Akt pathway is an important driver of the regulation of both normal and malignant mammary SC/progenitor cell numbers (11,34). The low frequency of mammospheres precluded our confirmation of the coincident increase in PTEN protein levels with transcripts, however, we have shown previously the coordinate induction of PTEN messenger RNA and protein by GEN treatment in mammary epithelial cells (22,25).

Bioactive concentrations of GEN at the nanomolar range are physiologically relevant; indeed, although a single oral GEN dose of 460 mg administered to humans resulted in peak plasma levels of 20 μM GEN, the levels that reached target tissues were significantly lower and within the nanomolar range (38,39). Based on the latter, we used 40 nM and 2 μM GEN in the present study. We observed that the lower GEN dose (40 nM) consistently attenuated primary and secondary mammosphere formation of transformed cell lines and primary epithelial cells isolated from mammary tumors; in contrast, the supra-physiological GEN dose (2 μM) was less effective in eliciting a similar biological outcome. For the MDA-MB-231 $CD44^+/CD24^-/ESA^+$ subpopulation, which is highly enriched for CSC, however, both doses of GEN were equally effective in suppressing mammosphere formation. Furthermore, although the lower GEN dose highly induced PTEN transcript levels in mammospheres formed from the more differentiated MCF-7 cells expressing PTEN (22), the higher GEN dose displayed a more robust induction of PTEN expression in the $CD44^+/CD24^-/ESA^+$ subpopulation of MDA-MB-231 cells. An experimental explanation for the noted distinct dose-dependent responses is lacking at the present time, however, these maybe partly related to the reported pro-proliferative activity of high GEN concentrations (micromolar range) in mammary epithelial cells (44); and possibly, the higher sensitivity of the more differentiated luminal progenitor cells for PTEN induction by GEN. The robust inhibition of ERα expression by 2 μM but not by 40 nM GEN suggests effects on the differentiated progenitor cells, given that CSC lacked ER and PR expression (45,46). Thus, the apparent discrepancies in the dose-dependent effects of GEN on mammosphere formation and gene expression may be a function of the distinct responses to GEN of CSC and the niche cells that comprise the tumor bulk.

The present study also demonstrated that bioactive components in systemic circulation resulting from BB consumption can inhibit self-renewal of the CSC-like subpopulation. Although BB phytochemicals have been previously demonstrated to reduce cell proliferation, enhance apoptosis and prevent epithelial–mesenchymal transition of breast cancer cell lines (47), the findings reported here are novel for several reasons. First, the inhibition of mammosphere formation by sera from BB-fed mice, whereas an *ex vivo* measurement, provides support for future feeding trial in rodent models to assess *in vivo* efficacy of consuming BB and other fruits with similar polyphenolic profiles to limit CSC self-renewal. Second, polyphenolic acid metabolites in BB such as hippuric acid may serve as candidate agents for targeting SC, suggesting their value as supplements for women being treated for the disease. Finally, higher BB consumption leading to supra-physiological levels of several polyphenolic acids may not be necessarily beneficial, as shown by the loss of functional response with the highest dose tested. Interestingly, the selected mixture of polyphenolic acids evaluated here preferentially inhibited mammosphere formation of the highly metastatic breast cancer cell line MDA-MB-231 and of the $CD44^+/CD24^-/ESA^+$ subpopulation of MDA-MB-231 cells and was ineffective in MCF-7 cells and primary epithelial cells isolated from Wnt-Tg tumors. This was convincingly demonstrated for MCF-7 cells cotreated with BB+GEN, wherein sphere formation inhibitory activity of the treatments could be solely attributed to 40 nM GEN. Since BB-sera was effective in inhibiting mammosphere formation in both cell lines, data collectively suggest that other phenolic acids or bioactive components targeting the luminal progenitor subpopulation were missing in the BB phenolic acid mixture used in this study. BB phytochemicals have been shown to exert their anti-growth and antimetastatic actions through modulation of the PI3K/Akt/NFκB pathway in MDA-MB-231 cells (47), however, inhibition of mammosphere formation reported here for hippuric acid did not appear to involve PTEN. Taken together, these findings imply that hippuric acid may exert its actions through mechanisms distinct from those of

the isoflavone GEN and invoke the participation of other SC/progenitor signaling pathways, the latter consistent with the recent demonstration that total loss of PTEN was insufficient to promote anchorage-independent growth of mammary epithelial cells (48).

In conclusion, this study demonstrates that bioactive components in foods conferring dietary benefits to the human population and which are highly bioavailable after regular consumption, can limit the expansion of SC-like and progenitor cells that promote tumor development, progression and recurrence. Whereas earlier studies have reported on the anti-inhibitory effects of dietary factors on breast cancer SC expansion (49,50), our results are the first to demonstrate that dietary factors may display selectivity in inhibiting mammosphere formation and by extension, SC renewal from distinct breast cancer subtypes. Our studies will facilitate the identification of dietary factors for the design of novel targeted therapies for potential translation in the clinics.

Supplementary material

Supplementary Table 1 can be found at http://carcin.oxfordjournals.org/.

Funding

This work was supported by the United States Department of Agriculture grant [CRIS 6251-5100002-06S, Arkansas Children's Nutrition Center to R.C.M.S.]; Arkansas Children's Hospital Children's University Medical Group (award to R.C.M.S.); Department of Defense Breast Cancer Research Program Concept Award (W81XWH-08-1-0548 to R.C.M.S.) and Department of Defense Breast Cancer Research Program Pre-doctoral fellowship (W81XWH-10-1-0047 to O.M.R.).

Acknowledgements

We thank Dr F.A.Simmen, Dr J.-R.Chen and laboratory members for helpful discussions and critical reading of this manuscript.

Conflict of Interest Statement: None declared.

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Received July 6, 2011; revised December 12, 2011; accepted December 27, 2011

Carcinogenesis vol.34 no.2 pp.464–474, 2013
doi:10.1093/carcin/bgs353
Advance Access publication November 8, 2012

Suppression of Wnt1-induced mammary tumor growth and lower serum insulin in offspring exposed to maternal blueberry diet suggest early dietary influence on developmental programming

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Despite the well-accepted notion that early maternal influences persist beyond fetal life and may underlie many adult diseases, the risks imposed by the maternal environment on breast cancer development and underlying biological mechanisms remain poorly understood. In this study, we investigated whether early exposure to blueberry (BB) via maternal diet alters oncogene Wnt1-induced mammary tumorigenesis in offspring. Wnt1-transgenic female mice were exposed to maternal Casein (CAS, control) or blueberry-supplemented (CAS + 3%BB) diets throughout pregnancy and lactation. Offspring were weaned to CAS and mammary tumor development was followed until age 8 months. Tumor incidence and latency were similar for both groups; however, tumor weight at killing and tumor volume within 2 weeks of initial detection were lower (by 50 and 60%, respectively) in offspring of BB- versus control-fed dams. Dietary BB exposure beginning at weaning did not alter mammary tumor parameters. Tumors from maternal BB-exposed offspring showed higher tumor suppressor (*Pten* and *Cdh1*) and lower proproliferative (*Ccnd1*), anti-apoptotic (*Bcl2*) and proangiogenic (*Figf*, *Flt1* and *Ephb4*) transcript levels, and displayed attenuated microvessel density. Expression of *Pten* and *Cdh1* genes was also higher in mammary tissues of maternal BB-exposed offspring. Mammary tissues and tumors of maternal BB-exposed offspring showed increased chromatin-modifying enzyme Dnmt1 and Ezh2 transcript levels. Body weight, serum insulin and serum leptin/adiponectin ratio were lower for maternal BB-exposed than control tumor-bearing offspring. Tumor weights and serum insulin were positively correlated. Results suggest that dietary influences on the maternal environment contribute to key developmental programs in the mammary gland to modify breast cancer outcome in adult progeny.

Introduction

The concept that the risk of adult diseases is partially established in the womb (i.e. fetal origin of adult diseases) was initially advanced by Professor David Barker for coronary heart disease in the 1980s (1). Since this seminal (and highly provocative) report, many epidemiological studies have linked poor maternal health to subsequent detrimental health effects in adult offspring (2–4). Nevertheless, the mechanisms underlying these life-long linkages, by which limited exposure (less than a year) to a non-supportive maternal environment can affect susceptibility to many devastating adult diseases, such as diabetes and cancer, are essentially unknown (5).

Breast cancer is the second leading cause of cancer-related deaths and the most common malignancy among women (6). Although this complex disease exhibits prominent genetic underpinnings (7), there is growing acceptance that susceptibility to breast cancer may be partly predetermined *in utero* (8). Initial support for this concept came from findings of increased breast cancer risk in daughters of women who used the synthetic estrogen diethylstilbestrol during pregnancy to prevent miscarriage (9,10). Diet and nutrition are well-accepted modifiable risks for breast cancer. Maternal exposure to dietary components has been reported to elicit significant effects on mammary gland development and breast cancer outcome in offspring in rodent models. For example, female rat offspring exposed to a maternal high fat diet during gestation have higher incidence of and larger mammary tumors when administered the carcinogen 7,12-dimethylbenz[*a*] anthracene as young adults (11,12). A fat-enriched maternal diet similarly increased spontaneous mammary tumor formation in outbred mice (13) and in transgenic mice overexpressing the *c-neu* oncogene (14). Protein restriction during pregnancy and lactation resulted in early onset of mammary tumorigenesis (by 2-fold) in rat offspring after *N*-methyl-*N*-nitrosourea administration (15). Further, agouti mice exposed to soy isoflavone genistein early in life via maternal diet were more likely to adopt a 'healthy' pseudoagouti rather than the 'disease-prone' yellow agouti phenotype (16). Our own studies showed that *in utero* and lactational exposure to a soy protein diet increased the latency and decreased the multiplicity of *N*-methyl-*N*-nitrosourea–induced mammary tumors in rat offspring (17). These protective effects were partly mediated by increased expression of the tumor suppressor E-cadherin in the mammary gland prior to carcinogenic insult. Nonetheless, whereas pharmacological agents, carcinogens and other environmental endocrine disruptors (e.g. high estrogen concentrations) are known to disrupt the normal course of mammary gland development via their gene-altering activities (18), a clear understanding of how maternal nutritional history and specifically, how maternal consumption of diets readily available to the Western population may predispose to breast cancer risk in progeny upon fetal exposure, is yet to be achieved.

To further explore the role of maternal diet in breast cancer outcome of adult progeny following fetal/neonatal exposure and potential underlying mechanisms, we evaluated the effects of whole blueberry (BB) powder consumed by pregnant and lactating dams on mammary tumor incidence and progression in their offspring. Our study used MMTV-Wnt1-transgenic (Wnt1-Tg) mice that overexpress the oncogene Wnt1 in the mammary gland (19). Although mutations in Wnt components are rarely seen in women with breast cancer, with the exception of metastatic breast carcinoma (20), overexpression of Wnt1 leads to increased β-catenin nuclear pools, a feature of >50% of human breast carcinomas (21). In a previous study, we showed enhanced prepubertal mammary gland differentiation in female rat offspring of dams consuming BB diet (22), although mammary tumor parameters were not evaluated. Recently, dietary intake of whole BB powder was shown to elicit anti-tumor and anti-metastatic activity in nude mice when transplanted with MDA-MB-231 triple negative breast cancer cells (23). Further, studies in humans (24) and rodents (25) demonstrated that dietary BB supplementation inhibited obesity-associated insulin resistance, in part by decreasing inflammation. In this study, we tested the hypothesis that fetal and lactational exposure

Abbreviations: Angpt1, angiopoietin 1; BB, blueberry; CAS, casein; Ccnd1, cyclin D1; Cdh1, cadherin 1; Col4a3, collagen type IV, alpha 3; DNMT, DNA methyl transferase; Ephb4, Eph receptor B4; EZH2, enhancer of zeste homolog 2; Figf, c-fos induced growth factor; Flt1, FMS-like tyrosine kinase 1; HDAC, histone deacetylase; MG, mammary gland; Mmp, matrix metallopeptidase; PND, postnatal day; PTEN, phosphatase and tensin homologue deleted on chromosome ten; PW, postweaning; QPCR, quantitative real-time polymerase chain reaction; TEB, terminal end bud; Thbs, thrombospondin; Timp, tissue inhibitor of metalloproteinase; Vegf, vascular endothelial growth factor; WT, wild-type; Wnt1-Tg, Wnt1-transgenic.

to maternal BB-supplemented diet will suppress mammary tumor incidence and/or progression in adult offspring through effects on critical oncogenic and metabolic (insulin) signaling pathways, and which may involve altered expression of key DNA-modifying enzymes.

Materials and methods

Animals and diets

Animal studies were performed in compliance with protocols approved by the University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee. Animals were housed in polycarbonate cages under conditions of 24ºC, 40% humidity and a 12 h light, 12 h dark cycle. Wnt1-Tg male mice [B6SJL-Tg(Wnt1)1Hev/J] were purchased from Jackson Laboratories (Bar Harbor, ME) and mated with wild-type (WT) females of the same strain to generate female Wnt1-Tg offspring. In Study 1, WT dams were randomly assigned to one of two diets prior to mating: (i) American Institute of Nutrition-93G-based pelleted diet containing casein (CAS) as the major protein source (Harlan, Indianapolis, IN) and (ii) CAS to which was added freeze-dried whole wild BB (*Vaccinium angustifolium*) powder (generous gift of the Wild Blueberry Association of North America, courtesy of Susan Davis) at 3% by weight of feed (designated BB). Supplementary Table 1, available at *Carcinogenesis* Online describes the preparation and processing of the composite BB powder, its average total anthocyanin content and relative anthocyanin concentrations. Diet with added BB powder was formulated to be isoenergetic and isonitrogenous (Supplementary Table 2, available at *Carcinogenesis* Online). Dams were maintained on the same diets throughout pregnancy and lactation. At weaning (PND21), female pups from both diet groups were weighed and genotyped for presence or absence of Wnt1-transgene following protocols suggested by the supplier (Jackson Laboratories). Dams fed either diet had comparable pregnancy weight gains, litter size and pup weaning weights (data not shown). A cohort of the WT and Wnt1-Tg female offspring from each diet group (n = 5–6 per group, per genotype) was euthanized at PND21 for analyses of mammary tissue parameters and gene expression (below). The remainder of the WT females were weaned to CAS (control) diet and at PND50, mammary tissues were collected for similar analyses (n = 5–6 per diet group per genotype). Corresponding Wnt1-Tg female offspring from the maternal diet groups (CAS: n = 33; BB: n = 28) were weaned to CAS and followed for mammary tumor development until age 8 months. In Study 2, WT dams mated to Wnt1-Tg males were fed CAS throughout pregnancy and lactation. Female Wnt1-Tg offspring were weaned to either CAS (n = 33; same group from Study 1) or BB-supplemented CAS (n = 22) diets and followed for mammary tumor development until age 8 months. Mice were provided food and water *ad libitum* and weight gains were documented weekly. All animals that did not develop tumors by 8 months of age were euthanized.

Mammary tissue collection and tumor analyses

Mammary gland pairs 2, 3 and 4 were harvested from PND21 (WT and Wnt1-Tg) and PND50 (WT) mice and used for analyses as follows. The left inguinal mammary gland (4) was fixed for whole-mount analyses, whereas the corresponding right gland (with lymph nodes removed) was immediately homogenized in Trizol (Invitrogen, Carlsbad, CA) for RNA analyses (below). The right-halves of mammary glands 2 and 3 were snap frozen at –80ºC for western blot analyses. Wnt1-Tg mice were palpated twice weekly from 4 weeks of age to monitor tumor formation. Tumors were measured using a caliper for length, width and height at initial detection and 2 weeks later were excised, weighed and re-measured. Tumor volume was calculated using the standard calculation for a sphere ($4/3 \times 3.14 \times ab^2$) (26). Tumors were divided into three parts: one part was processed for immunohistochemistry and pathology, the second was extracted for RNA analyses and the third was stored at –80ºC for protein analyses.

Microvessel density analysis

Microvessel density was determined in tumors from Wnt1-Tg mice (n = 3–4 per diet group) as described previously (27). Microvessels were immunolabeled with rat anti-mouse CD34 (AbD-serotec, Kidlington, UK) at 1:25 dilution, followed by biotinylated rabbit anti-rat IgG at 1:100 dilution (Vector Laboratories, Burlingame, CA). Staining was visualized using VectaStain Elite ABC kit followed by Vector DAB substrate kit as per manufacturer's instructions (Vector Laboratories). Sections were lightly stained with hematoxylin. Stained tissue sections were scanned by Aperio ScanScope (Aperio Technologies, Vista, CA) to capture high resolution digital images of the entire section. Vessels were counted in five random areas of tumor infiltration (1 mm^2/area) per tumor sample in a blinded fashion.

Whole-mount analyses, immunohistochemistry and tumor pathology

Whole mounts of fat pads were prepared as described (22) and evaluated under a dissecting microscope. The total numbers of terminal end buds (TEBs), located at the leading edge of the fat pad, were counted from whole mounts of the left mammary gland 4; four to five mice were used for each diet group. Branching density for each gland was quantified by counting the number of branched points within a box of fixed dimensions (22). Histopathology of tumors from hematoxylin–eosin stained paraffin sections (17) was determined by a residency-trained veterinary pathologist (L.J.H.) using accepted criteria established by an expert panel of surgical, veterinary and experimental pathologists (28). Immunostaining for cyclin D1 used anti-cyclin D1 antibody (1:250 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) following the manufacturer's protocols. Sections of solid carcinoma tumors from four individual mice per diet group were analyzed. Negative controls were tumor sections processed in parallel, except with the omission of primary antibody.

Quantitative real-time PCR

Total RNA isolation, preparation of corresponding cDNAs and quantitative real-time PCR (QPCR) analyses followed previously described protocols (29). Primers (Supplementary Table 3, available at *Carcinogenesis* Online) were designed to span introns, using Primer Express software (Applied Biosystems, Foster City, CA) and were synthesized by Integrated DNA Technologies (Coralville, IA). The expression of each target mRNA was calibrated to a standard curve generated using pooled cDNAs and normalized to that of TATA-box binding protein (*Tbp*).

Western blot analyses

Whole cell lysates and nuclear extract proteins were prepared from frozen mammary tumors and subjected to immunoblotting as described previously (30). Primary antibodies used were anti-PTEN (1:1000; Cell Signaling, Danvers, MA), anti-cyclin D1 (1:400; Santa Cruz Biotechnology), anti-DNMT1 (1:1000; Abcam, Cambridge, MA), anti-EZH2 (1:1000; Cell Signaling) and anti-β-actin (1:2000; Sigma Chemical Co., St Louis, MO). The immunoreactive proteins were visualized with the Amersham ECL Plus kit (GE Healthcare Life Sciences, Piscataway, NJ). Digital images were captured using the GE Image Scanner III detection system and quantified using Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Serum hormone assays

The levels of insulin and leptin in sera of Wnt1-Tg mice displaying solid carcinoma tumors 2 weeks post initial tumor detection (Study 1: n = 9 individual mice per diet group) were measured using the Milliplex MAP mouse serum adipokine kit (Millipore Corp., Billerica, MA), following the manufacturer's instructions. For insulin and leptin assays, the sensitivity thresholds were 24.9 and 3.7 pg/ml, respectively, whereas intra- and interassay coefficients of variations were <4.5 and 10.3%, respectively. Serum insulin levels for mice in Study 2 (n = 8–9 individual mice per diet group) were measured using the Rat/Mouse Insulin ELISA kit (Millipore); sensitivity was 0.2 ng/ml and intra- and interassay coefficients of variations were 5 and 11%, respectively. Serum adiponectin concentrations were measured using a mouse adiponectin ELISA kit (Millipore Corp.). Adiponectin assay sensitivity was 0.5 ng/ml and intra- and interassay coefficients of variations were <6%. Serum IGF-1 levels were measured using the Mouse/Rat IGF-1 immunoassay kit (R&D Systems, Minneapolis, MN), with a sensitivity threshold of 1.6 pg/ml.

Data analyses

Data are presented as mean ± SEM and were compared by t-test or one-way analysis of variance, using SigmaStat version 3.5 Software (SPSS, Chicago, IL). Differences in tumor incidence between diet groups were determined by Fisher Exact test. $P \leq 0.05$ was considered to be statistically significant.

Results

Dietary exposure to BB through maternal diet altered mammary parameters in offspring

Whole mounts of fat pads were prepared from WT and Wnt1-Tg female offspring of dams that were fed control (CAS) or BB-supplemented (BB) diets during pregnancy and lactation (Figure 1A). At weaning (PND21), mammary glands from Wnt1-Tg offspring of CAS-fed dams displayed extensive ductal side-branching and highly developed lobuloalveoli structures (Figure 1B). In contrast, mammary glands from WT littermates (Figure 1B) showed rudimentary branching and distinct TEBs, with no detectable alveoli. The extensive mammary hyperplasia

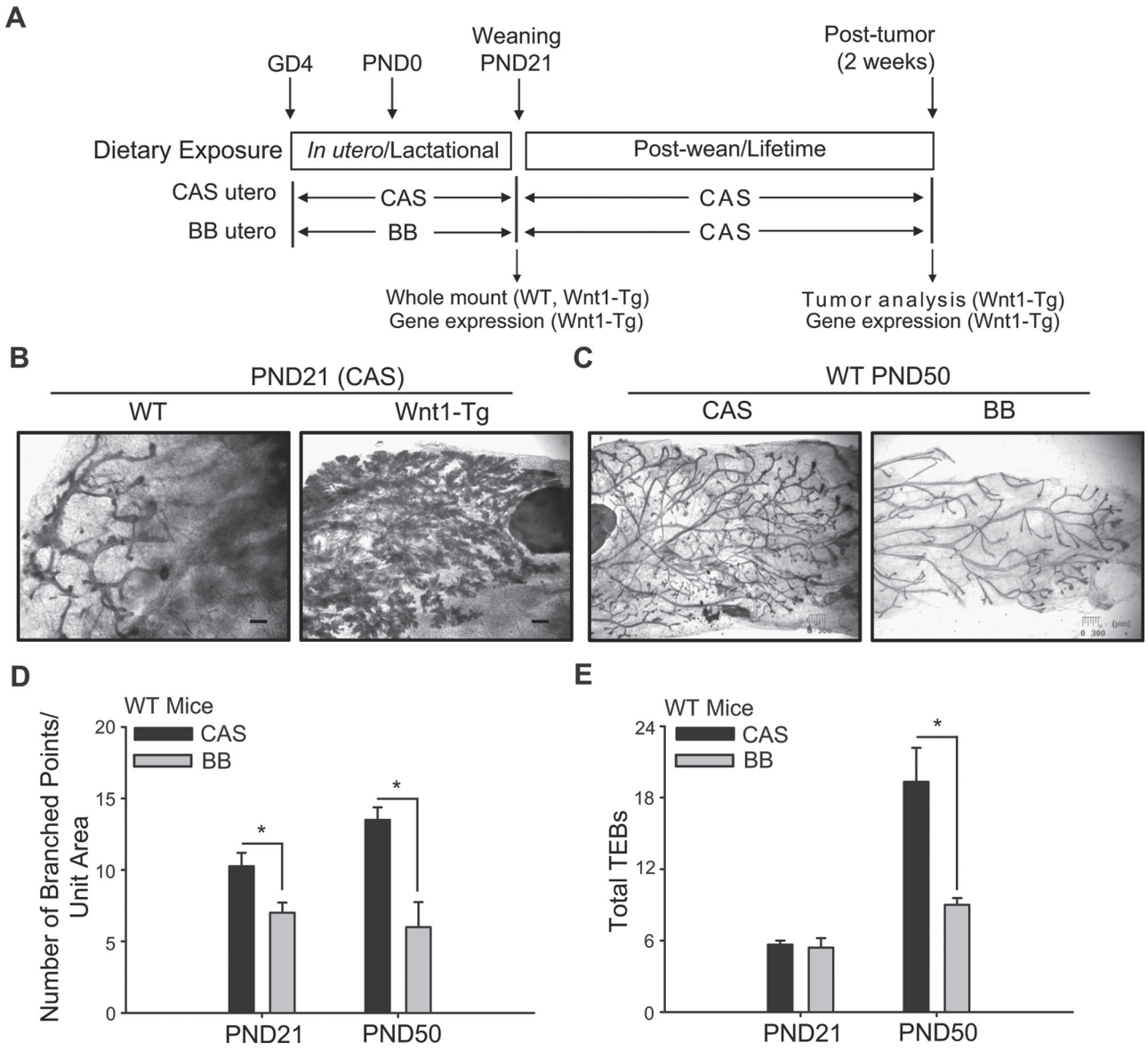

Fig. 1. *In utero* and lactational exposure to BB reduces ductal branching and TEB numbers in offspring. **(A)** Dietary regimen. Wild-type (WT) dams were mated with Wnt1-Tg males. Dams were fed control (CAS) diet or the BB-supplemented CAS diet from mating through pregnancy and lactation. Wnt1-Tg and WT female pups were weaned to CAS diet. Inguinal mammary gland (4) was collected for RNA (right MG) and whole mount (left MG) from weaning (PND21) pups. For the tumor study, Wnt1-Tg female offspring that were exposed via maternal diet to either CAS ($n = 33$) or BB ($n = 28$) were monitored weekly by palpation for tumor development. Two weeks post-tumor detection, serum was collected and tumors were harvested for RNA and protein analyses. **(B)** Whole-mount analysis revealed excessive branching and hyperplasia in mammary glands of Wnt1-Tg female pups at weaning (PND21), compared with WT mice, as previously reported (19). **(C)** Shown are the mammary glands of PND50 virgin WT female mice exposed to CAS or BB via maternal diet. **(D)** Ductal branching was quantified in PND21 and PND50 WT mice. **(E)** Quantification of mammary TEB in PND21 and PND50 WT mice exposed to maternal CAS or BB diets. Results are mean ± SEM; $^*P < 0.05$ relative to CAS ($n = 5$ mice per diet, per PND). *Bar*, 300 μm. 3× magnification.

elicited by Wnt1 overexpression in PND21 Wnt1-Tg offspring precluded an accurate determination of differences in mammary branching and TEB numbers with diet. To determine whether maternal diet affected mammary gland development, whole mounts of fat pads from young adult (PND50) WT female offspring that were exposed to maternal CAS or BB diets were evaluated. Dietary BB-exposed PND50 WT offspring displayed mammary glands that had less side-branching than CAS-exposed counterparts (Figure 1C). The numbers of branched points (a measure of the ductal network) in mammary glands of BB offspring were lower than those of control offspring at both PND21 and PND50 (Figure 1D). The numbers of TEBs were comparable for mammary glands of offspring of the two diet groups at PND21 and were lower in mammary glands of BB-exposed offspring relative to those of control offspring at PND50 (Figure 1E).

Dietary exposure in utero and at lactation to BB inhibited mammary tumor growth in offspring

Female Wnt1-Tg offspring of dams that were fed CAS or BB diets during pregnancy and lactation were followed for mammary tumor onset up to age 8 months. Exposure to maternal BB diet did not alter mammary tumor incidence in these females, as determined by Kaplan–Meier analysis (Figure 2A). The average age of tumor onset (5–6 months) was comparable ($P = 0.265$) between offspring of both diet groups (Figure 2B). However, the change in tumor volume, defined as the percentage change in tumor size from initial detection to tumor harvest at 2 weeks postdetection, and tumor weights (measured at tumor harvest and normalized to body weights) were lower for offspring of the BB-fed dams (Figure 2C and D). Histopathological analyses of the tumors showed

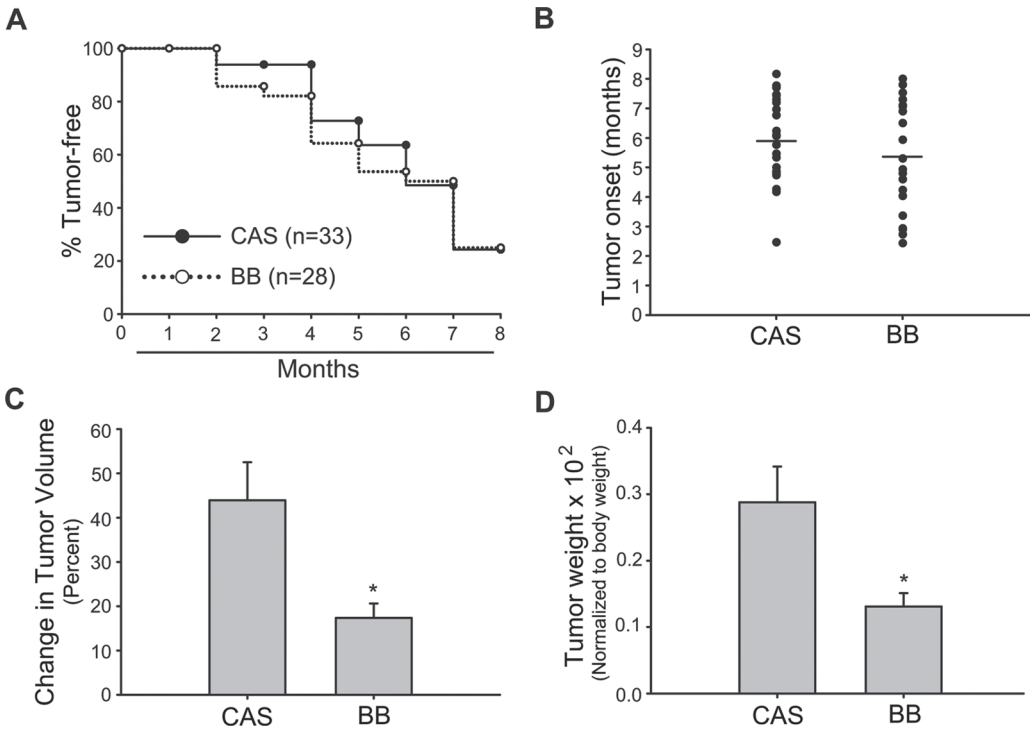

Fig. 2. Maternal intake of BB suppresses tumor growth in Wnt1-Tg female offspring. Tumor incidence **(A)**, onset **(B)**, change in tumor volume **(C)** and tumor weight at killing **(D)** were compared for Wnt1-Tg female offspring of CAS- or BB-fed dams. Presence of tumors was monitored by weekly visual inspection and palpation from 1 to 8 months of age. The mean tumor onset was calculated as the age of initial tumor appearance. Tumor weight was normalized to body weight. Change in tumor volume was calculated as described in Materials and methods. Results are mean ± SEM; $*P < 0.05$ for BB relative to CAS.

predominance of solid carcinoma (28) (Supplementary Table 4, available at *Carcinogenesis* Online).

Dietary BB exposure postweaning did not alter mammary tumor parameters

To determine if postweaning exposure to BB relative to the control diet, recapitulated the effects of early BB exposure through maternal diet, female Wnt1-Tg offspring of control (CAS) diet-fed dams were weaned (at PND21) to CAS or BB diets and monitored for tumors until age 8 months (Supplementary Figure 1A, available at *Carcinogenesis* Online). The incidence, onset, change in volume and weights of mammary tumors of Wnt1-Tg females fed BB diets did not differ from those of Wnt1-Tg females fed CAS (Supplementary Figure 1B–3E, available at *Carcinogenesis* Online). Diet did not affect the type of mammary tumors that developed (Supplementary Table 4, available at *Carcinogenesis* Online).

Gestational + lactational exposure to BB diet promoted tumor suppressor expression in mammary tumors

Solid carcinoma tumors from Wnt1-Tg offspring of dams that were fed CAS or BB diets (n = 9/diet group) were evaluated for expression of tumor suppressor (*Pten*; *p53*; cadherin 1, *Cdh1*), proproliferative (Cyclin D1, *Ccnd1*; *c-Myc*) and anti-apoptotic (*Bcl2*, *survivin*) genes by QPCR. Transcript levels of *Pten* and *Cdh1* were increased, whereas those of *Ccnd1* and *Bcl2* were decreased, in tumors of maternal BB-exposed offspring (Figure 3A). Diet did not influence expression of *Wnt1*, *p53*, *c-Myc* and *survivin* in tumors. The changes in gene expression for *Pten* and *Ccnd1* in mammary tumors with exposure to maternal BB diet were confirmed at the protein level by western (Figure 3B and C) and for *Ccnd1*, also by immunohistochemistry (Figure 3D). The majority (80%) of tumors analyzed in the BB group by immunohistochemistry had low to undetectable cyclin D1 protein (Figure 3D).

Maternal exposure to BB diet decreased microvessel density in mammary tumors of Wn1-Tg offspring

To determine whether the lower tumor volume and weight seen with maternal exposure to BB diet (Figure 2C and D) could involve regulation of growth factor supply via the vasculature, tumor microvessel density was analyzed by CD34 immunostaining (Figure 4A). CD34-reactive microvessel numbers were 2-fold lower (CAS = 57.72 ± 12.1 versus BB = 28.04 ± 2.85; $P = 0.039$) in tumors from Wnt1-Tg mice exposed to maternal BB diet relative to the CAS-exposed group (Figure 4B).

To evaluate possible mediators of the observed decrease in microvessel formation with maternal dietary BB exposure (Figure 4A and B), we measured the transcript levels of a panel of genes with known anti- and proangiogenic activities, in mammary tumors of Wnt1-Tg offspring. Tumors from offspring exposed to maternal BB diet relative to those exposed to control diet had higher expression of genes *Col4a3* and *Thbs1*, which were shown previously to inhibit angiogenesis (31,32); no differences for *Thbs2*, *Timp1* or *Timp2* transcript levels were observed between the dietary groups (Figure 4C). Analysis of proangiogenic genes revealed suppression of *Ephb4* (33), *Figf* (34) and *Flt1* (34) transcript levels in tumors from offspring with maternal BB exposure (Figure 4D). Similar changes in the expression of other proangiogenic genes such as *Angpt1*, *Mmp2*, *Mmp9*, *Vegfa* and *Vegfc* were not observed (Figure 4D).

Early exposure to BB diet altered gene expression of DNA methylation and chromatin-modifying enzymes in mammary tumors

To examine potential associations between maternal BB dietary effects on offspring mammary tumor outcome and epigenetic mechanisms, the expression patterns of a select group of DNA methylation and chromatin-modifying enzyme genes were evaluated in mammary tumors by QPCR. Transcript levels of DNA methyl transferase 1 (*Dnmt1*) and Enhancer of zeste homolog 2 (*Ezh2*) were higher in tumors from BB-exposed offspring (Figure 5A); these changes in transcript abundance were confirmed at the protein level by western blots (Figure 5B). In contrast, transcript levels of enzymes involved

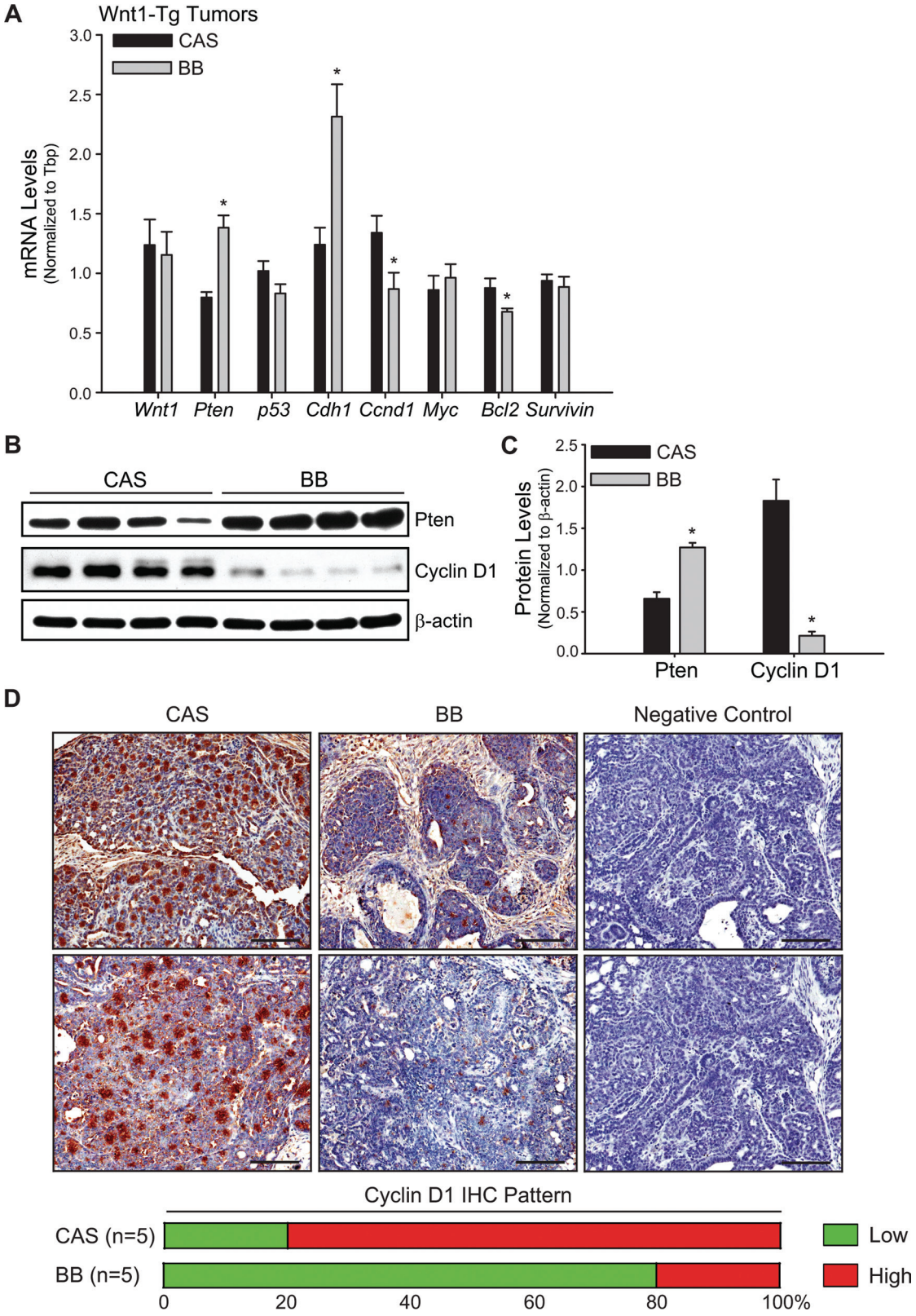

Fig. 3. Tumors from Wnt1-Tg mice exposed to BB via maternal diet exhibit higher expression of anti-proliferative, proapoptotic and prodifferentiation markers. **(A)** Genes involved in cell proliferation, apoptosis, differentiation and tumor progression were evaluated by QPCR in tumors from Wnt1-Tg mice exposed to CAS or BB diets *in utero* and during lactation. *Tbp* was used as a normalizing control; $*P < 0.05$ relative to CAS ($n = 9$ per diet group). **(B)** Western blot analysis of PTEN and cyclin D1 proteins in CAS or BB tumors. Each lane (50 µg of total protein) represents an individual animal. **(C)** Immunoreactive bands (in B) were quantified by densitometry. Normalized values relative to β-actin are presented as histograms ($*P < 0.05$ relative to CAS). **(D)** Representative cyclin D1 immunostaining of CAS and BB tumors from five tumored mice (per diet group) is shown at ×200 magnification. Each *panel* for CAS and BB represents solid carcinoma tumor sections (Supplementary Table 4, available at *Carcinogenesis* Online) from an individual mouse. Negative control shows lack of immunostaining in the absence of primary antibody. *Bar*, 100 µm.

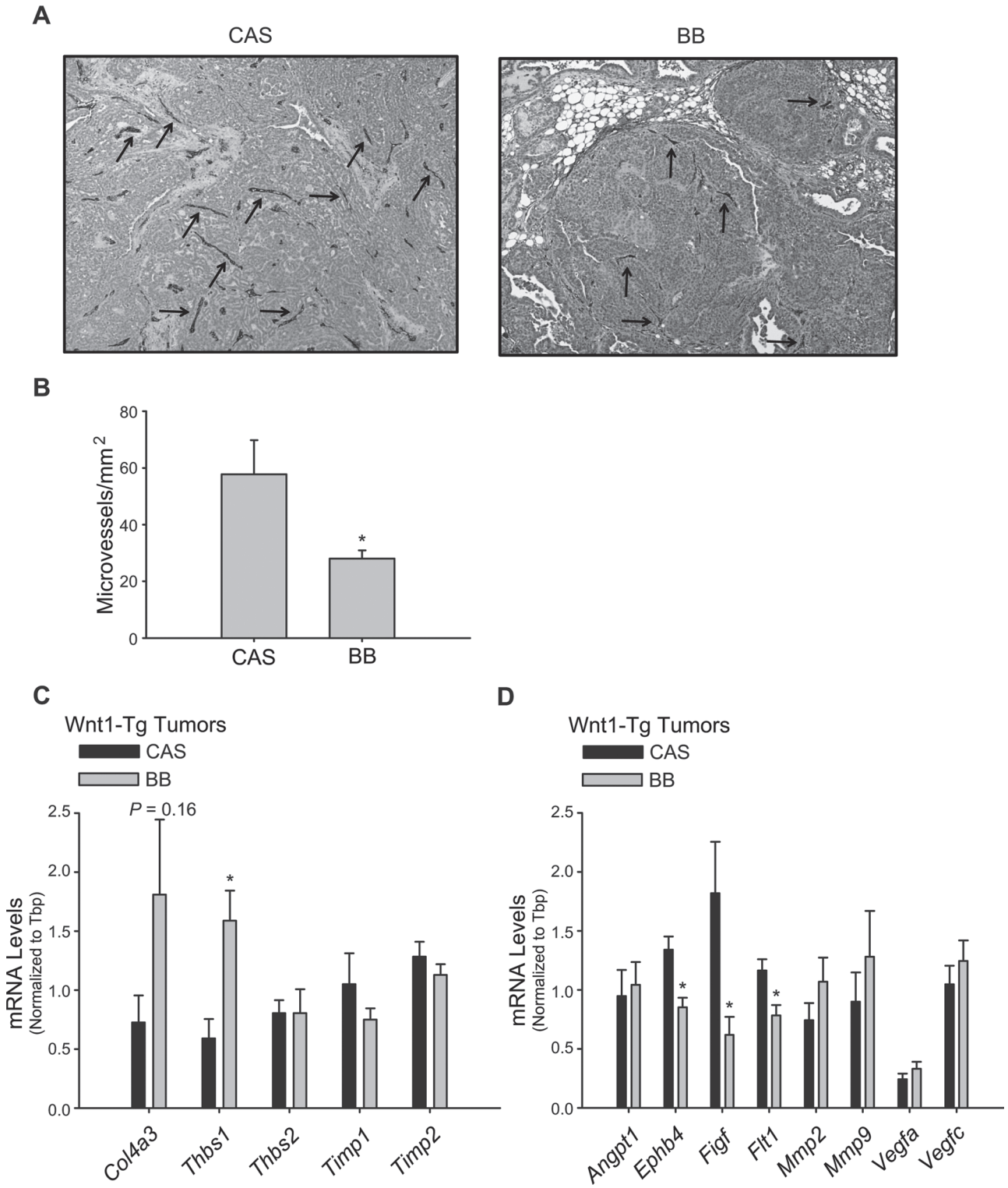

Fig. 4. Maternal exposure to BB diet decreases microvessel density in mammary tumors of Wnt1-Tg offspring. **(A)** Representative images of CD34 staining (arrows) of microvessels in mammary tumors of Wnt1-Tg mice fed CAS or BB via maternal diet. **(B)** CD34-immunoreactive vessels were quantified in five random areas per tumor sample ($n = 3–4$ independent tumors per diet group); $^*P < 0.05$ relative to CAS. Anti-angiogenesis **(C)** and proangiogenesis **(D)** transcript levels in mammary tumors from Wnt1-Tg offspring exposed to CAS or BB via maternal diet. mRNA levels were determined by QPCR and normalized to *Tbp* ($n = 8–10$ mice per diet group); $^*P < 0.05$ relative to CAS, for each gene analyzed.

in histone modifications namely histone deacetylases 1, 2, 3 and 9 (*Hdac1*, *2*, *3 and 9*) were not influenced by diet (Figure 5A).

Preneoplastic mammary tissues of PND21 Wnt1-Tg female offspring of the two diet groups were similarly evaluated for changes in expression of tumor suppressor *Pten* and *Cdh1*, proproliferative *Ccnd1* and DNA/chromatin-modifying enzyme (*Dnmt1*, *Ezh2*, *Hdac3*, *6 and 9*) genes, by QPCR. The increase in *Pten*, *Cdh1*, *Dnmt1* and *Ezh2* transcript levels in mammary tumors of maternal BB-exposed offspring was also observed in corresponding non-tumor PND21 mammary tissues (Figure 5C and 5D). In contrast to mammary tumors,

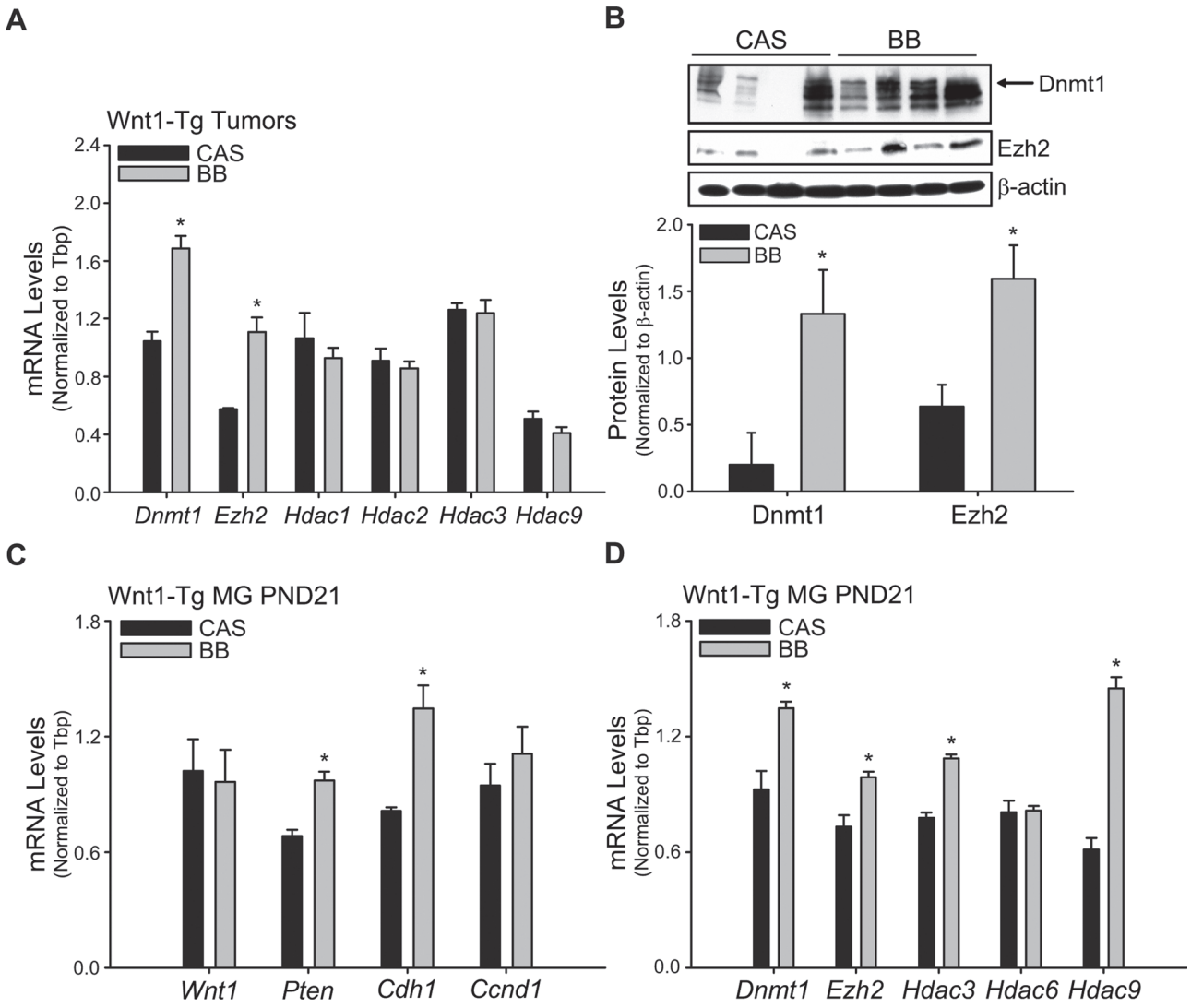

Fig. 5. Gene expression of DNA methylation and chromatin-modifying enzymes in mammary tumors and in non-tumor (preneoplastic) mammary glands. **(A)** Gene expression of DNA methylation (*Dnmt1* and *Ezh2*) and histone deacetylation (*Hdac1*, *Hdac2*, *Hdac3* and *Hdac9*) enzymes was quantified by QPCR in tumors from Wnt1-Tg mice exposed to maternal CAS or BB diets. *Tbp* was used as a normalizing control; **P* < 0.05 relative to CAS (*n* = 9 per diet group). **(B)** *Top*, western blot analyses of DNMT1 (arrow) and EZH2 proteins (50 µg) in tumors from maternal CAS or BB diet groups; each lane represents an individual animal. *Bottom*, immunoreactive bands were quantified by densitometry and normalized to β-actin; relative values are presented as histograms (**P* < 0.05 relative to CAS). **(C)** Gene expression of proliferative (cyclin D1, *Ccnd1*) and proapoptotic/prodifferentiation (*Pten*; E-cadherin, *Cdh1*) markers was evaluated in mammary glands of Wnt1-Tg mice at weaning (PND21). **(D)** Gene expression of DNA methylation (*Dnmt1* and *Ezh2*) and histone deacetylation (*Hdac3*, *Hdac6* and *Hdac9*) enzymes in mammary glands of weaned Wnt1-Tg mice exposed to CAS or BB via maternal diets. *Tbp* was used as a normalizing control; **P* < 0.05 relative to CAS (*n* = 6 per diet group).

preneoplastic mammary tissue *Ccnd1* transcript levels did not differ, whereas those for *Hdac3* and *Hdac9* were greater with maternal BB exposure. Diet did not alter *Wnt1* gene expression in tissues (Figure 5C), as shown for mammary tumors (Figure 3A).

Maternal diet influenced body weight and hormone levels in tumored offspring

To determine if maternal diet affected endocrine parameters that may influence mammary tumor development long after the dietary exposure, body weights of offspring were evaluated throughout the study, and serum levels of the adipokines leptin and adiponectin and of insulin and IGF-1 were measured in tumored mice at killing. Although body weights of offspring from the two diet groups did not differ at weaning, lower body weights were found for maternal BB-exposed Wnt1-Tg females by PND50 and which persisted for lifetime, when compared with maternal CAS-exposed counterparts (Figure 6A). BB-exposed, tumor-bearing offspring showed numerically lower serum leptin/adiponectin ratio (Figure 6B), and significantly reduced serum insulin levels (Figure 6C). Serum IGF-1 levels did not differ for offspring of the two groups (CAS: 443.47 ± 23.75 ng/ml; BB: 426.11 ± 52.19 ng/ml). Tumor weights were positively correlated with serum insulin levels (R = 0.46; Figure 6D). Interestingly, although mice exposed to BB diets beginning at postweaning had lower body weights than those of CAS-fed mice (Figure 6E), serum insulin levels for mice in these two groups did not differ (Figure 6F).

Discussion

This study provides strong support for the significant influence of maternal diet on mammary tumor outcome in progeny. Using the Wnt1-Tg mouse model of spontaneous human breast cancer and BB powder intake at 3% of the diet, amounts which are readily achievable with half to one cup of BBs per day for an average-weight individual, we showed that maternal BB consumption during pregnancy

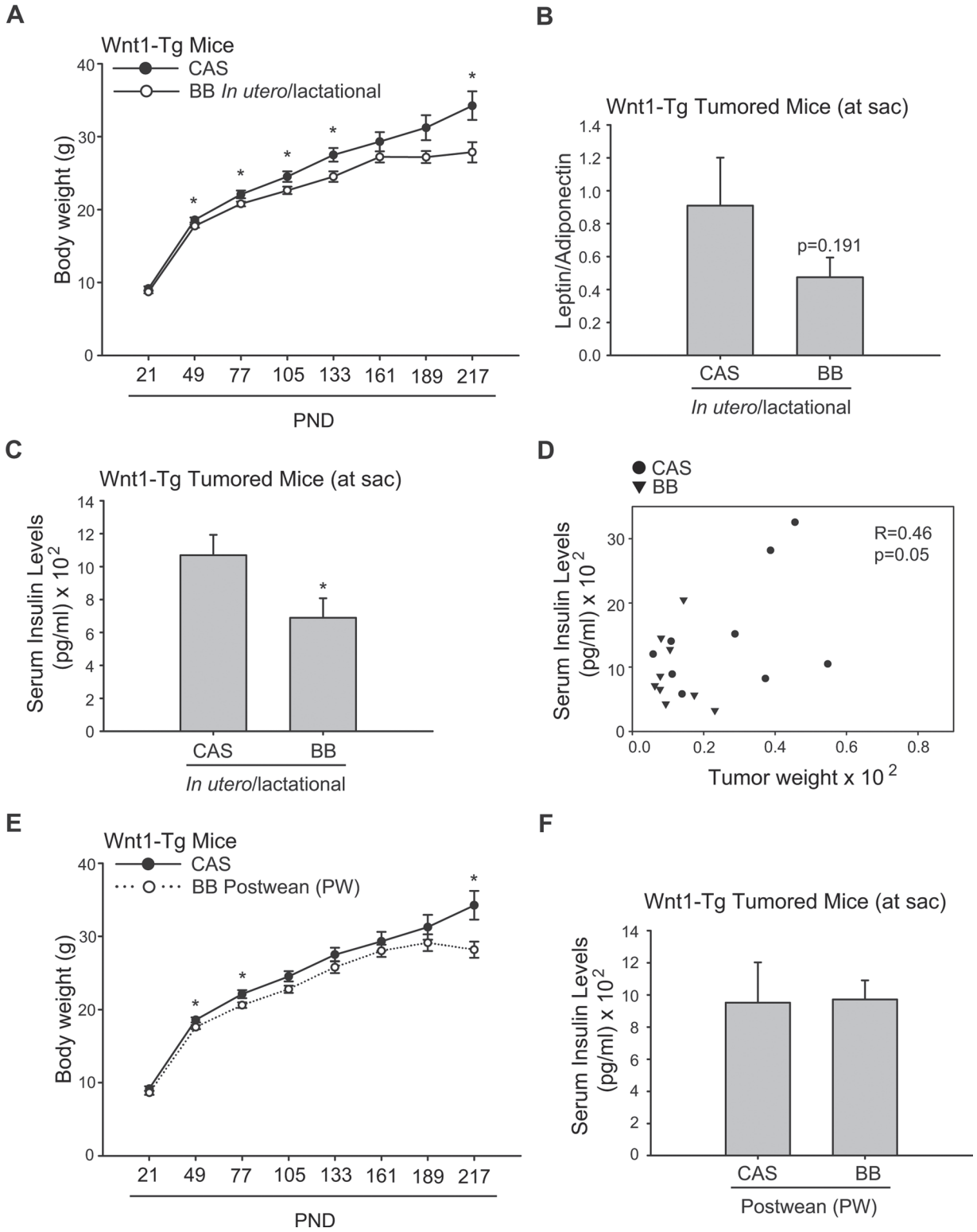

Fig. 6. Maternal BB exposure lowers body weight and suppresses serum insulin levels in Wnt1-Tg offspring. **(A)** Body weights of Wnt1-Tg mice enrolled in the tumor study were recorded monthly from weaning (PND21) until study conclusion (up to 8 months). *$P < 0.05$ relative to CAS. **(B and C)** The ratio of serum leptin to adiponectin levels and serum insulin levels, respectively, were measured for tumor-bearing Wnt1-Tg mice at killing as described in Materials and methods ($n = 9$ per diet group). **(D)** Correlation between serum insulin and tumor weight (normalized to body weight) in Wnt1-Tg tumored mice exposed to CAS (circle) or BB (triangle) via maternal diet. The Pearson correlation coefficient is 0.46; $P = 0.05$. **(E)** Body weights and **(F)** serum insulin levels for mice fed CAS or BB diets beginning at weaning until killing. *$P < 0.05$ relative to CAS.

and lactation elicited reductions in mammary tumor weight and tumor volume in adult progeny, resulting in a more favorable prognosis. The suppression of mammary tumor growth with early BB exposure, which was not recapitulated when the same BB diet was introduced during early childhood (postweaning) and continued through adulthood, suggests that the effects of early BB exposure with later adult

consequence, probably occurred during an early window of mammary gland development. We further showed that the inhibitory effects of early BB exposure on mammary tumor growth are associated with: (i) elevated expression of tumor suppressors PTEN and E-cadherin and lower expression of anti-apoptotic Bcl2 and proliferative cyclin D1, genes in mammary tumors; (ii) an early increase (at weaning) of PTEN and E-cadherin gene expression in mammary tissues, which was accompanied by reduced ductal side-branching in developing (PND21) and young adult (PND50) and lower TEB numbers in young adult (PND50) mammary glands, respectively, and (iii) decreased intratumoral microvessel density and decreased and increased expression, respectively, of a subset of pro- and anti-angiogenic genes. The differences in ductal branching with early BB exposure are not likely due to differing estrous cycle stage at the time of tissue collection because these were observed even at PND21, when these mice have not begun to cycle. Moreover, we previously demonstrated that exposure to BB through maternal diet did not promote sexual maturity (22). We provided evidence to suggest that the effects of early dietary BB exposure on gene expression patterns in preneoplastic mammary tissues and mammary tumors may involve epigenetic modifications through early BB effects on EZH2 and DNMT1 expression, both of which regulate the expression of many developmental genes (36). Finally, we showed that the long-term effects of maternal BB diets are associated with altered metabolic parameters in offspring, manifested as decreased body weights, lower serum insulin levels and diminished leptin/adiponectin ratio, all of which are negatively linked to breast cancer progression (37–39).

In this study, we utilized Wnt1-Tg female mice as an *in vivo* model for human breast cancer because numerous targets of the Wnt signaling pathway are overexpressed or deregulated in breast tumors (40). Moreover, we have previously demonstrated that tumor suppressors PTEN and E-cadherin, which are linked by their regulation of β-catenin degradation and nuclear transport (41,42), are *in vivo* and *in vitro* targets of diets/dietary factors with breast cancer protective effects (29,30,43,44). Although maternal BB diet did not reduce Wnt1-induced tumor incidence, most likely because highly activated Wnt signaling is a powerful driver of breast cancer initiation (19) and activation of this pathway could not be inhibited or reversed by bioactive components present in BB, limited early exposure to this diet was sufficient to alter the trajectory of mammary tumor volume/weight in offspring. The latter suggests the protective effects of early exposure to BB on pathways involved in tumor progression. The lack of a similar 'rescue' of overactivated Wnt signaling by provision of the BB diet at postweaning and through adulthood, suggests that a critical developmental window of mammary gland development is affected by maternal nutrition. The latter possibility places the maternal (*in utero*/lactational) environment as a highly relevant nutritional target for breast cancer prevention (45).

Although this study did not address the *in utero*/lactational mechanism(s) and the precise mediators by which maternal BB exposure influences offspring mammary gene expression and hence, breast cancer risk and outcome, it is possible that maternal BB intake may reduce oxidative stress, limit the inflammatory milieu and oppose mitogenic signals within the uterine microenvironment of the developing fetus, thereby leading to metabolic changes with later consequences. In this regard, BB polyphenolic acid metabolites, which are highly bioavailable, are known to display high anti-oxidative potential (46). Further, BB intake has been recently shown to negatively affect proinflammatory gene networks involving IL-13 and IFNγ and to suppress Wnt/β-catenin signaling pathways through its induction of Wnt inhibitor SFRP4 (23). In a recent study, we demonstrated that several BB polyphenolic acids present in sera can inhibit mammosphere formation of human breast cancer cells, the latter a measure of mammary stem cell activity (47). These findings raise the interesting possibility that stem cell pathways in developing mammary glands may be influenced by early BB exposure through maternal diets. Maternal obesity and high fat/high calorie maternal diets have been shown to impose a proinflammatory state on the fetus (48), and the latter has been suggested to underlie some of the adult chronic diseases associated with metabolic syndrome in adult offspring of obese dams (12,13). Although we did not find differences in body weights of dams consuming CAS or BB diets, similar to a previous report in rats (22), the observed lower body weights, serum insulin levels and serum leptin/adiponectin ratio in adult offspring of dams fed BB diet, relative to those of dams fed CAS, are consistent with suppression of a proinflammatory environment associated with obesogenic conditions, with maternal BB intake. Interestingly, pups exposed to BB diets beginning only at postweaning, although exhibiting lower body weights than those fed CAS, did not manifest the lower insulin levels attributed to early (pre- and postnatal) BB exposure. Thus, life-long effects of maternal BB consumption in the suppression of mammary tumor progression in adult offspring may be attributed to developmental programming of insulin sensitivity as well as of anti-inflammatory response, both of which can be mediated by epigenetic mechanisms, to counter the negative effects of adipogenesis and insulin resistance.

Formation of new blood vessels (i.e. angiogenesis) is essential for breast cancer progression, and increased microvessel density in breast carcinoma has been correlated with tumor size, grade and lymph node metastasis (49). Our findings of decreased microvessel density and alterations in the expression of a subset of genes known to inhibit angiogenesis (31–35) in mammary tumors of Wnt1-Tg offspring that were exposed to maternal BB relative to control diet indicate that early BB exposure could modulate intratumoral angiogenic factor levels. The latter possibility provides a testable mechanism (to be addressed in future studies) by which maternal dietary BB intake may influence tumor outcome in women predisposed to breast cancer due to aberrant activation of Wnt signaling.

Bioactive dietary factors have been reported to be potent modifiers of the epigenome, exerting influence on the expression and/or activity of numerous chromatin-modifying enzymes (50–52). Herein, we found that exposure to BB through maternal diet increased the expression of *Dnmt1*, *Ezh2* and *Hdac*3 and *Hdac*9 genes in non-tumor (preneoplastic) mammary tissues of weaning offspring and these effects persisted for DNMT1 and EZH2 in mammary tumors. Although these findings implicate changes in DNA methylation and histone modifications as important for the protective effects of maternal BB exposure in offspring mammary tumor outcome, the endogenous gene targets of these epigenetic changes remain unclear. An earlier study reported that dietary intake of black raspberries by patients with colon tumors led to decreased DNMT1 protein levels and reduced methylation (and hence, activation) of anti-proliferative p16 and Wnt suppressor genes (53). Paradoxically, inhibition of mammary tumor progression by maternal BB exposure was associated in this study with higher PTEN levels coincident with increased DNMT1 and EZH2 expression. In other studies, increased methylation of PTEN gene promoter by DNMT1 (54) and EZH2 (55) has been reported to result in lower PTEN expression and higher Akt activity, leading to increased breast cancer invasion and metastasis (56). Although we have no experimental data to explain our results at the present time, we suggest that dietary exposure under distinct contexts (e.g. tumor versus normal, non-tumor) leading to similar DNA methylation status might elicit different effects. Indeed, it is worth noting that an increase in DNA methylation may be beneficial under some contexts (16). In that study, *in utero* exposure to bisphenol A, which caused DNA hypomethylation in mouse offspring, was counteracted by supplementation of maternal diet with folic acid or the phytoestrogen genistein, leading to DNA hypermethylation. Thus, some early epigenetic marks persisting to adulthood in mammary tumors may be beneficial. Further studies to identify changes in global and promoter-specific methylation events elicited by BB in mammary tissues and tumors using more sophisticated procedures of DNA methylation profiling will address these apparent discrepancies.

In conclusion, our use of the Wnt1-Tg mouse model of human breast cancer has provided compelling support for the maternal (*in utero*/lactational) environment of the developing mammary gland as a modifiable determinant of breast tumor growth/progression in adult progeny. In light of the life-long effects of the limited (maternal only) dietary exposure described here, the notion of pubertal mammary

development as most susceptible to 'tumorigenic insults' may need reconsideration. Given the many health risks that can be imposed by obesity and poor dietary choices of pregnant and nursing mothers on their progeny, further studies on nutritional epigenomics and programming and how these may underlie breast cancer outcome and influence treatment of the disease are warranted.

Supplementary material

Supplementary Tables 1–4 and Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/

Funding

United States Department of Agriculture (CRIS-6251-5100002-06S, Arkansas Children's Nutrition Center to R.C.M.S); Department of Defense Breast Cancer Research Program Pre-doctoral Fellowship (W81XWH-10-1-0047 to O.M.R.); National Institutes of Health-National Cancer Institute (RO1 CA136493 to F.A.S.); University of Arkansas for Translational Research Institute/Arkansas Breast Cancer Research Program (1UL1RR029884).

Conflict of Interest Statement: None declared.

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Received June 29, 2012; revised October 8, 2012; accepted October 31, 2012